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ORIGINAL ARTICLE

Mechanisms of xylanase-induced nitric oxide and phosphatidic acid production in tomato cells

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Abstract The second messenger nitric oxide (NO), phosphatidic acid (PA) and reactive oxygen species (ROS) are involved in the plant defense response during plant–pathogen interactions. NO has been shown to participate in PA production in response to the pathogen-associated molecular pattern xylanase in tomato cell suspensions. Defense responses downstream of PA include ROS production. The goal of this work was to study the signaling mechanisms involved in PA production during the defense responses triggered by xylanase and mediated by NO in the suspension-cultured tomato cells. We analyzed the participation of protein kinases, guanylate cyclase and the NO-mediated posttranslational modification S-nitrosylation, by means of pharmacology and biochemistry. We showed that NO, PA and ROS levels are significantly diminished by treatment with the general protein kinase inhibitor staurosporine. This indicates that xylanase-induced protein phosphorylation events might be the important components leading to NO formation, and hence for the downstream regulation of PA and ROS levels. When assayed, a guanylate cyclase inhibitor or a cGMP analog did not alter the PA accumulation. These results suggest that a cGMP-mediated pathway is not involved in xylanase-induced PA formation. Finally, the inhibition of protein S-nitrosylation did not affect NO formation but compromised PA and ROS production. Data collectively indicate that upon xylanase perception, cells activate a protein kinase pathway required for NO formation and that, S-nitrosylation-dependent mechanisms are involved in downstream signaling leading to PA and ROS.

Keywords Lipid signaling · Nitric oxide · Pathogen-associated molecular pattern · Plant defense response · Reactive oxygen species · Tomato

Abbreviations

8-Br-cGMP	8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt monohydrate
AU	Arbitrary unit
CDPK	Ca^{2+}-dependent protein kinase
CFM	Cell free medium
DAF-FM-DA	3-Amino,4-aminomethyl-2′,7′-difluorescein diacetate
H_2DCF-DA	2′,7′,-Dichlorofluorescein diacetate
LY83583	6-Anilino-5,8-quinolinequinone
MAPK	Mitogen-activated protein kinase
NEM	*N*-Ethylmaleimide
NO	Nitric oxide
PA	Phosphatidic acid
PAMP	Pathogen-associated molecular pattern
PD098059	2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PLC	Phospholipase C
PLD	Phospholipase D
ROS	Reactive oxygen species
SB202190	4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole
SPL	Structural phospholipids
TFP	Trifluoperazine dihydrochloride
TLC	Thin layer chromatography

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Introduction

Plants are constantly challenged by different pathogens. In order to resist these pathogens they can activate a battery of responses or defense mechanisms 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 that are referred to as elicitors. Xylanases are a widespread group of extracellular enzymes involved in plant cell infection by pathogens. They are produced by a plethora of microorganisms including the saprophytic and phytopathogenic bacteria, and fungi (Sunna and Antranikian 1997; Beg et al. 2001). Xylanases from the fungus *Trichoderma* spp. are well-known pathogen-associated molecular pattern (PAMP) elicitors of defense responses in various plants such as tobacco and tomato (Bailey et al. 1990; Avni et al. 1994). Downstream signal transduction cascades become activated upon recognition of xylanase, which include production of nitric oxide (NO), phosphatidic acid (PA) and reactive oxygen species (ROS), extracellular medium alkalization, ethylene biosynthesis, mitogen-activated protein kinase (MAPK) activation, pathogenesis-related protein expression and hypersensitive cell death (Laxalt et al. 2007).

One of the second messengers reported to participate in the plant defense response is the lipid PA (Laxalt and Munnik 2002). PA has been shown to accumulate upon treatment with the PAMPs xylanase, *N*-acetylchitotetraose, chitosan and flagellin in tomato cells (van der Luit et al. 2000; Laxalt et al. 2007; Raho et al. 2011), as well as the *N*-acetylchitooligosaccharide in rice cells (Yamaguchi et al. 2003). PA has also been shown to accumulate upon treatment with the race-specific elicitor Avr4 in Cf-4 expressing tobacco cells (de Jong et al. 2004), and during AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis* (Andersson et al. 2006). Nod factors have been reported to induce PA in alfalfa cells (den Hartog et al. 2003). It has been well documented that PA is able to trigger the ROS formation (the so called oxidative burst) in plants (Testerink and Munnik 2005), and PA can activate NADPH oxidase in *Arabidopsis* (Zhang et al. 2009).

PA can be enzymatically generated via two pathways in plants. Phospholipase C (PLC) hydrolyzes the signaling phospholipid phosphatidylinositol 4,5-bisphosphate into two second messengers: inositol 1,4,5-trisphosphate which releases Ca^{2+} from intracellular compartments into the cytosol (Bootman et al. 2001), and diacylglycerol, which is rapidly phosphorylated through the action of the enzyme diacylglycerol kinase (DGK) generating PA (Testerink and Munnik 2005). Another source of PA is the enzyme phospholipase D (PLD) which catalyzes the hydrolysis of structural phospholipids, such as phosphatidylcholine, into PA (Wang 2000). The main PA enzymatic source in xylanase signaling is the activation of the PLC/DGK pathway (van der Luit et al. 2000; Laxalt et al. 2007). The study of regulation of the activity of PA-generating enzymes is an under-explored area. The activity of PLC and DGK enzymes is regulated by Ca^{2+} (Mueller-Roeber and Pical 2002) and may also be regulated by phosphorylation. A phosphoproteomic approach showed that AtPLC2 becomes phosphorylated after the elicitation of *Arabidopsis* cells with flagellin (Nühse et al. 2003). However, it is still unknown whether this posttranslational modification affects its activity.

Another ubiquitous second messenger in plants is NO. The mechanisms underlying the effect of NO have been studied by means of pharmacology and biochemistry. NO can exert its effects by: (i) posttranslational modification of target proteins by either direct S-nitrosylation of critical cysteine thiol group(s) or nitration of tyrosines (Stamler et al. 2001; Schopfer et al. 2003), (ii) activation of the enzyme guanylate cyclase (GC) that synthesizes cGMP and downstream regulates cytosolic Ca^{2+} concentration via cADPR (Gow and Ischiropoulos 2001), (iii) activation of MAPKs, Ca^{2+}-dependent protein kinases (CDPKs), and members of the SNF1-related kinase type 2 family (Pagnussat et al. 2004; Lanteri et al. 2006; Lamotte et al. 2006). NO is involved in the plant defense response of a growing list of plant–pathogen interactions (Distéfano et al. 2010). However, how NO activates phospholipid-related enzymes in the plant defense response has to be further analyzed. Recently, several targets of protein S-nitrosylation have been characterized during the hypersensitive response in *Arabidopsis* (Romero-Puertas et al. 2008). High local NO concentration and the presence of nitrosative agents in the membrane make S-nitrosylation a plausible regulatory mechanism for PA-generating enzymes. So far, there are no reports of in vivo or in vitro S-nitrosylation of these enzymes neither in animals nor in plants. Evidence is also accumulating for cGMP and protein kinases as important components of NO-related signal transduction during the plant–pathogen interactions (Durner et al. 1998; Clarke et al. 2000). Until now, it has not been established whether the cGMP, protein phosphorylation or S-nitrosylation-dependent mechanisms are involved in the xylanase-induced and NO-mediated signaling pathway.

We have shown that NO is required for PLC/DGK activation in xylanase-treated tomato cells (Laxalt et al. 2007). However, we do not know the mechanisms involved in xylanase-induced NO, PA and ROS production. In this context, the goal of this work was to study the signaling mechanisms involved in PA production during the defense responses triggered by xylanase and mediated by NO in suspension-cultured tomato cells. We analyzed the participation of S-nitrosylation, protein phosphorylation and

cGMP, by means of pharmacology and biochemistry. We provide evidence that phosphorylation events participate in the xylanase-induced signaling cascade leading to NO production and S-nitrosylation-dependent mechanisms are involved in downstream signaling to PA and ROS formation.

Materials and methods

Cell suspensions

Suspension-cultured tomato cells (*Solanum lycopersicum* cv. Money Maker, line Msk8) were grown at 25°C in the dark at 125 rpm in Murashige & Skoog medium containing vitamins (Duchefa, Haarlem, The Netherlands) supplemented with 5.4 μM naphthalene-1-acetic acid and 1 μM 6-benzyladenine as described earlier (Laxalt et al. 2007). Tomato cells were kindly gifted by Dr. G. Felix (University of Tuebingen, Germany).

Chemicals

All the pharmacological compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used according to the manufacturer's instructions. *N*-Ethylmaleimide (NEM) is a cell permeable, sulfhydryl alkylating agent that covalently modifies cysteine residues thereby preventing S-nitrosylation (Ahern et al. 2002). *S*-Methyl methanethiosulfonate (MMTS) is an another cell permeable reagent used to block thiol groups in vivo in a highly efficient, specific and reversibe manner (Pascual et al. 1997; Khodakhah et al. 1998; Fu and Kirk 2001; Karala and Ruddock 2007). It has also been used for studying protein S-nitrosylation (Jaffrey et al. 2001; Foster and Stamler 2004). 6-Anilino-5,8-quinolinequinone (LY83583) is a competitive inhibitor of soluble GC. It lowers the production of cGMP levels in a wide range of tissues, with negligible effect on cAMP levels (Mulsch et al. 1988). 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt monohydrate (8-Br-cGMP) is a cell permeable non-hydrolyzable derivative of cGMP (Schwarzschild and Zigmond 1991). Staurosporine is a potent, cell permeable, broad spectrum inhibitor of protein kinases (Rüegg and Burgess 1989). 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD098059) is a specific and cell permeable inhibitor of MAPK kinases (Alessi et al. 1995). 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) is a highly selective, potent, reversible, competitive and cell permeable inhibitor of mammalian p38 MAPKs. It binds to both active and inactive forms of the enzyme and is an important tool for the study of p38 MAPK function both in vivo and in vitro (Ward et al. 2000). Trifluoperazine dihydrochloride (TFP) is a calmodulin antagonist that was reported to inhibit plant CDPKs by competing with the binding of Ca^{2+} to their calmodulin-like domain (Chico et al. 2002). Xylanase from *Trichoderma viride* was purchased from Fluka (Buchs, Switzerland). The fluorescent probes 3-amino,4-aminomethyl-2′,7′-difluorescein diacetate (DAF-FM-DA) and 2′,7′,-dichlorofluorescein diacetate (H_2DCF-DA) were purchased from Molecular Probes (Eugene, OR, USA). Reagents for lipid extractions and subsequent analysis, as well as silica 60-TLC plates were purchased from Merck (Darmstadt, Germany).

Treatments

Suspension-cultured tomato cells of 4–5 days old were exposed to different treatments and for different time periods in 2 ml reaction vials (for PA production assays) or in Greiner 96-well plates (for NO and ROS production assays). Inhibitor treatments were performed by incubating the cells 30 min prior to the treatment. The concentrations of the inhibitors were chosen according to the literature. Inhibitors were dissolved in the corresponding solvent or water as indicated by the manufacturer, and diluted in cell-free medium (CFM) to reach the final concentration. In all experiments, the appropriate solvent control (ethanol or dimethylsulfoxide) was used as a comparison. Maximal solvent concentrations were 0.025% (v/v) ethanol or 0.05% (v/v) dimethylsulfoxide.

$^{32}P_i$ phospholipid labeling, extraction and analysis

Eighty-five of microliters of tomato cells was labeled for 3 h with 185 kBq $^{32}P_i$ (PerkinElmer, Waltham, MA, USA) prior to treatment with 200 μg ml^{-1} xylanase and/or different pharmacological compounds for 30 min. Control treatments were performed by adding CFM. Incubations were stopped by adding 20 μl of 50% perchloric acid. Lipids were extracted by adding 3.75 volumes of $CHCl_3$:MeOH:HCl (50:100:1, by vol.) and processed as described before (Laxalt et al. 2007). Lipids were separated on heat-activated thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) employing an ethyl acetate (EtAc) solvent system as a mobile phase composed of EtAc:iso-octane:HCOOH:H_2O (12:2:3:10, by vol.). The EtAc TLC system was specifically used to separate PA from the other phospholipids. Radioactivity was visualized and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA). PA levels were quantified as a ratio against the structural phospholipid (SPL) levels (PA/SPL) and expressed as a fold increase taking PA levels of control cells as 1. For statistical analysis, One Way ANOVA test

was used as appropriate. A value of $P < 0.05$ was considered significant for mean differences.

Quantification of NO and ROS levels

Endogenous production of NO or ROS was monitored by incubating 95 µl batches of cultured tomato cells with DAF-FM-DA or H_2DCF-DA (final concentrations of 1 µM or 4 µM, respectively) for 30 min at 25°C in the dark on Greiner 96-well plates. The cells were then treated with 20 µl CFM or CFM containing xylanase (final concentration of 200 µg ml^{-1}) in the absence or presence of different inhibitors as indicated in the figures. Fluorometric measurements were performed in a Fluoroskan Ascent microwell plate fluorometer (Thermo Electron Company, Vantaa, Finland) using Chroma (Chroma Technology Corp, Rockingham, VT, USA) filters D480-40 and D525-30 for excitation and emission, respectively. Fluorescence of each individual well was measured every 1 min over 20 ms at 25°C. At least three individual experiments were performed with three technical replicates each.

Data analysis and statistics

Where indicated, values are expressed as means ± SE of at least three independent experiments. For statistical analysis of the results, One Way ANOVA was used as appropriate, after testing for data normality. A value of $P < 0.05$ was considered significant for mean differences.

Results

Effect of S-nitrosylation, cGMP or protein phosphorylation inhibition on xylanase-induced PA accumulation

It was previously shown that xylanase triggers PA accumulation within minutes in tomato cell suspensions (van der Luit et al. 2000; Laxalt et al. 2007). To determine the putative mechanism for which xylanase induces PA production, we first applied the cell permeable sulfhydryl alkylating reagent NEM. NEM is considered as an inhibitor of S-nitrosylation since it covalently modifies cysteine residues (Ahern et al. 2002). We then characterized the formation of PA in xylanase-treated tomato cells by performing dose–response experiments with the inhibitor. Phospholipids were labeled by incubating the cells for 3 h with orthophosphoric acid ($^{32}P_i$) and subsequently treated for 30 min with 200 µg ml^{-1} xylanase in the presence or absence of different doses of NEM. Phospholipids were extracted and separated by TLC with an EtAc solvent system. Figure 1a (upper panel, representative for three independent experiments) shows a typical profile of the radiolabeled phospholipids obtained after 30 min of treatment. As visualized in Fig. 1a, most of the $^{32}P_i$ was incorporated into SPL and no significant changes in the levels of these lipids could be detected in any of the treatments performed. The radioactivity of the signaling lipid PA was quantified as a ratio against the radioactivity in total SPL (PA/SPL). Values were subsequently expressed as fold increase with respect to the control (Fig. 1a, lower panel). Figure 1a shows that the effect of xylanase in accumulating the PA was significantly suppressed by 100 µM NEM. We have done experiments with the solvent ethanol used to dissolve NEM. Results indicated that the treatment of tomato cells with 0.025% (v/v) ethanol, which is equivalent to the maximal solvent concentration achieved in 100 µM NEM, evoked no differences in PA production respect to CFM (control)-treated cells (data not shown). In conclusion, data indicate that S-nitrosylation events are involved in xylanase signaling leading to PA formation.

We then investigated whether the formation of PA is affected by the application of the competitive inhibitor of soluble GC LY83583. LY83583 lowers the production of cGMP levels in a wide range of tissues (Mulsch et al. 1988). We evaluated the effect of different doses of this compound on the level of PA induced by xylanase. Results presented in Fig. 1b showed that LY83583 did not modify PA accumulation in response to xylanase, whereas treatment with the cell permeable non-hydrolyzable cGMP analog 8-Br-cGMP resulted in a similar amount of PA as in control cells (Fig. 1c). These results suggest that a cGMP-mediated pathway is not involved in xylanase-induced PA formation.

To further investigate the mechanism underlying PA formation, we assayed the broad spectrum protein kinase inhibitor staurosporine (Rüegg and Burgess 1989) on this response. The effect of xylanase in accumulating PA was reversed by 0.1 µM and 1 µM staurosporine (Fig. 2a), indicating that the activation of protein kinases is required for the accumulation of PA by the elicitor xylanase in tomato cells. No significant changes in the accumulation of phospholipids could be observed when cells were treated with 0.05% (v/v) dimethylsulfoxide, which is the final concentration used in treatments with 1 µM staurosporine (data not shown).

We were interested in identifying the protein kinase(s) that may participate in xylanase signaling. Therefore, we studied the effect of different pharmacological compounds that block MAPK or Ca^{2+}-dependent protein kinase (CDPK) pathways on the regulation of PA

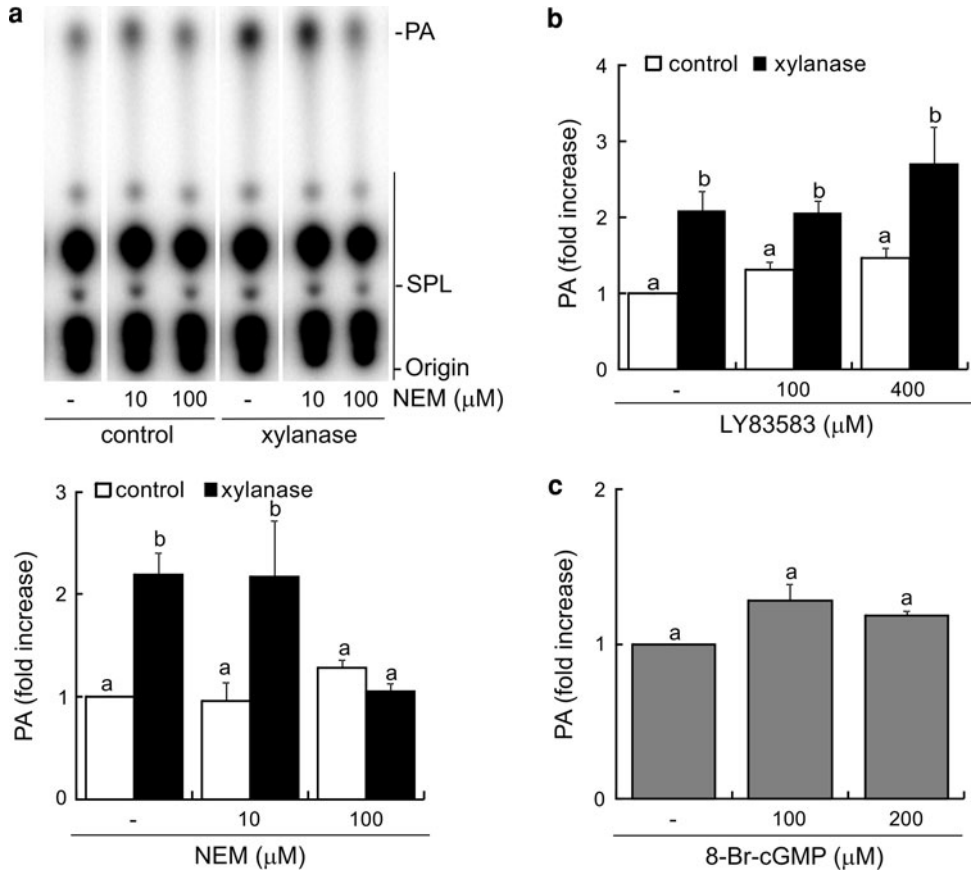


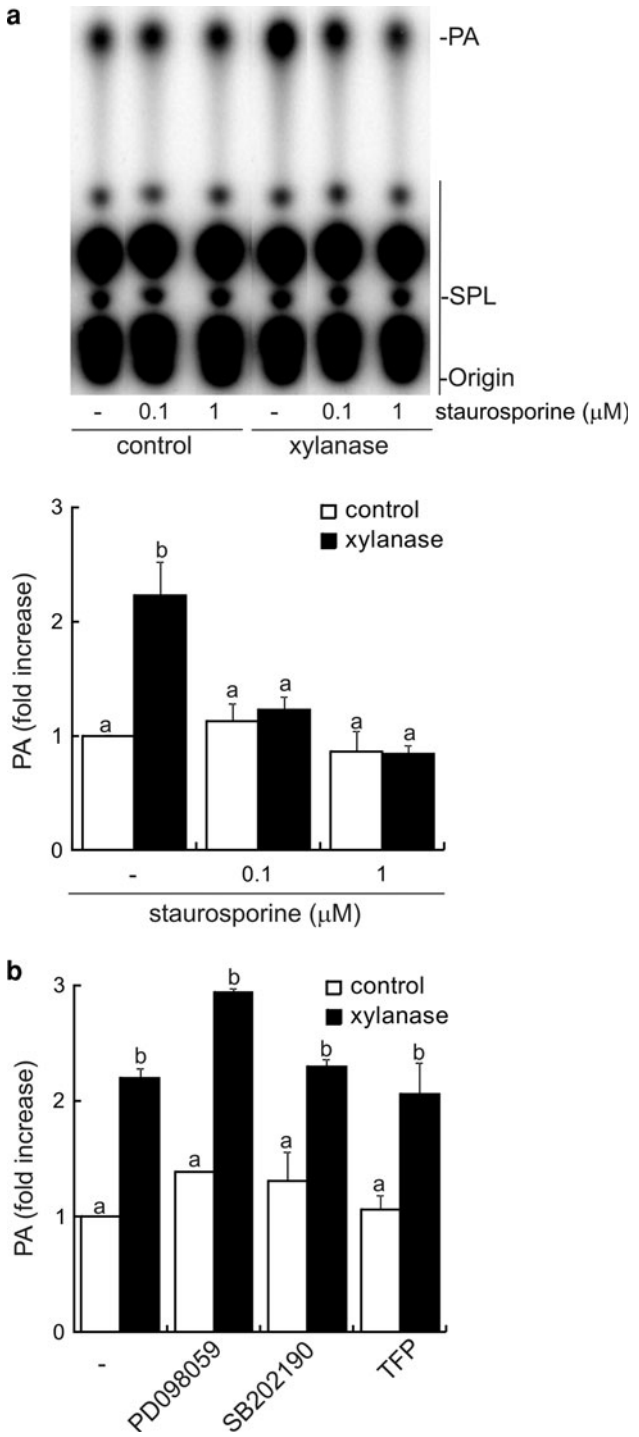
Fig. 1 Effect of the S-nitrosylation inhibitor NEM, the GC inhibitor LY83583 and the non-hydrolyzable cGMP 8-Br-cGMP on PA accumulation in tomato cells. Suspension-cultured tomato cells were labeled with $^{32}P_i$ for 3 h and then treated for 30 min with CFM (control) or with 200 μg ml^{-1} xylanase in the presence or absence of different doses of NEM (**a**), LY83583 (**b**), or treated with different doses of 8-Br-cGMP (**c**). Lipids were extracted and separated by TLC using an EtAc solvent system. Radioactivity was visualized and quantified by phosphoimaging. The quantification of three independent experiments is plotted. The radioactivity of the signaling lipid PA was quantified as a ratio against the radioactivity in total structural phospholipids (PA/SPL). PA levels are expressed as fold increase in relation to control the samples in the absence of pharmacological compound. *Error bars* indicate SE of the means. *Bars* with the same letter do not significantly differ at $P < 0.05$ according to One Way ANOVA

formation in xylanase-treated tomato cells. To test the participation of MAPK cascades we used the specific and cell permeable inhibitors of: (i) MAPK kinases PD098059 (Alessi et al. 1995) and (ii) mammalian p38 MAPKs SB202190 (Ward et al. 2000). To evaluate the involvement of CDPKs, we treated cells with the calmodulin antagonist TFP. TFP was reported to inhibit plant CDPKs by competing with the binding of Ca^{2+} to their calmodulin-like domain (Chico et al. 2002). As visualized in Fig. 2b, the application of all these inhibitors did not compromise PA production triggered by xylanase. Taking this evidence into account, we conclude that MAPK and CDPK pathways do not participate in PA accumulation in response to xylanase in tomato cells.

Altogether, data presented in Figs. 1 and 2 support a role for both protein S-nitrosylation and phosphorylation events in xylanase signaling leading to PA formation.

Staurosporine prevents xylanase-induced NO production in tomato cells

The elicitor xylanase triggers a rapid and sustained (over at least 30 min) production of NO in tomato cells (Laxalt et al. 2007). It has been shown that protein phosphorylation is needed for the NO accumulation in different experimental systems (Lamotte et al. 2004; Vandelle et al. 2006). Thus, we studied whether the inhibition of protein phosphorylation also affects xylanase-induced NO formation by quantifying the level of NO in treatments with staurosporine. A quantitative dose–response experiment was performed using a fluorometer and the NO specific probe DAF-FM-DA. NO specific fluorescence was monitored for 30 min in cells treated with 200 μg ml^{-1} xylanase and increasing concentrations of staurosporine (Fig. 3a, upper panel). The gradient of NO production throughout 30 min



◀ **Fig. 2** The general protein kinase inhibitor staurosporine blocks the formation of PA triggered by xylanase in tomato cells. Suspension-cultured tomato cells were labeled with $^{32}P_i$ for 3 h and then treated for 30 min with CFM (control) or with xylanase in the presence or absence of different doses of staurosporine (**a**) or 1 μM PD098059 (MAPK kinase inhibitor), 10 μM SB202190 (MAPK inhibitor) or 10 μM TFP (CDPK inhibitor) (**b**). Lipids were separated by EtAc TLC and quantified by phosphoimaging. PA levels are expressed as fold increase in relation to control the samples in the absence of inhibitor. *Error bars* indicate SE of the means ($n = 3$). *Bars* with the same letter do not significantly differ at $P < 0.05$ according to One Way ANOVA

was calculated and expressed as percentage, 100% corresponding to the arbitrary unit (AU) of xylanase-treated cells in the absence of the inhibitor (Fig. 3a, lower panel). Figure 3a shows that the production of NO in xylanase treatment was inhibited dose dependently by the application of staurosporine, with a 100% inhibition at 1 μM. Moreover, treatments with staurosporine alone did not affect the level of NO compared to CFM (control)-treated tomato cells (Fig. 3a). Control experiments performed with equivalent volume of the solvent dimethylsulfoxide [0.05% (v/v) final] showed no variation with respect to control cells (data not shown).

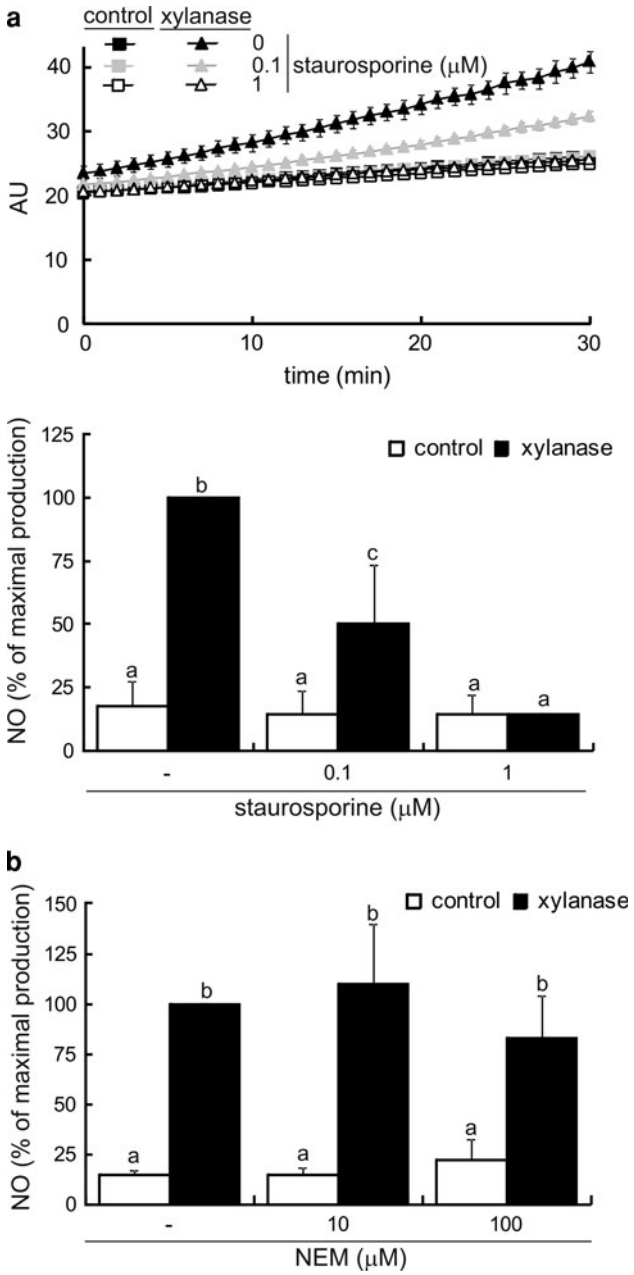
The application of specific inhibitors of MAPK cascades (PD098059 and SB202190) and of CDPKs (TFP) did not alter NO production triggered by xylanase (data not shown), suggesting that these pathways do not participate in xylanase-induced NO formation.

We then corroborated that the level of NO was not affected by the application of the S-nitrosylation inhibitor NEM. Suspension-cultured tomato cells were incubated with CFM (control) or with xylanase in the presence or absence of 10 and 100 μM NEM. As expected, Fig. 3b shows that the application of increasing concentrations of NEM did not compromise the formation of NO in response to the elicitor xylanase.

In conclusion, Fig. 3 indicates that the protein phosphorylation events are needed for NO production upon xylanase perception in tomato cells.

Xylanase-triggered ROS production is significantly inhibited by both staurosporine and NEM

ROS production is one of the earliest responses to pathogen attack and has been shown to occur following the xylanase elicitation (Laxalt et al. 2007). Laxalt et al. (2007) also suggested that NO lies upstream of PA, and PA lies upstream of ROS in xylanase-elicited tomato cells. We analyzed whether the level of ROS was affected by incubation with staurosporine. A quantitative dose–response experiment was performed using a fluorometer and the ROS specific probe H_2DCF-DA. ROS specific fluorescence was monitored over 30 min in cells treated with 200 μg ml^{-1} xylanase and increasing concentrations of staurosporine. Fluorescence values were expressed as AU (Fig. 4a, upper panel). The gradient of ROS production from 5 to 15 min was calculated and expressed as percentage, 100% corresponding to the AU of xylanase-treated cells in absence of the inhibitor (Fig. 4a, lower panel). ROS generation in response to xylanase was completely prevented by the application of 1 μM staurosporine



◀ **Fig. 3** Staurosporine prevents xylanase stimulation of NO formation. **a** Dose– and time–response curves of NO production. Cells were incubated with CFM (control) or with xylanase in the presence or absence of different doses of staurosporine (0.1 and 1 μM). NO level was determined over a 30 min period in a microwell-plate fluorometer using the fluorescent probe DAF-FM-DA (1 μM). Fluorescence values are expressed as arbitrary unit (AU). *Upper panel* a representative graph is shown. Error bars represent SE of the three technical replicates within the experiment. *Lower panel* the gradient of NO production throughout 30 min was calculated and expressed as percentage, 100% corresponding to the AU of xylanase-treated cells in absence of the inhibitor. Error bars represent SE of the means ($n = 3$). Bars denoted with the same letter do not significantly differ at $P < 0.05$ according to One Way ANOVA. **b** Cells were incubated with CFM (control) or with xylanase in the presence or absence of 10 or 100 μM NEM. NO level was determined and quantified as indicated in **a**. *Error bars* represent SE of the means ($n = 4$). *Bars* with the same letter do not significantly differ at $P < 0.05$ according to One Way ANOVA

(Fig. 4a). In addition, treatments with staurosporine alone or with the solvent dimethylsulfoxide showed no variation compared to control the cells (Fig. 4a and data not shown, respectively).

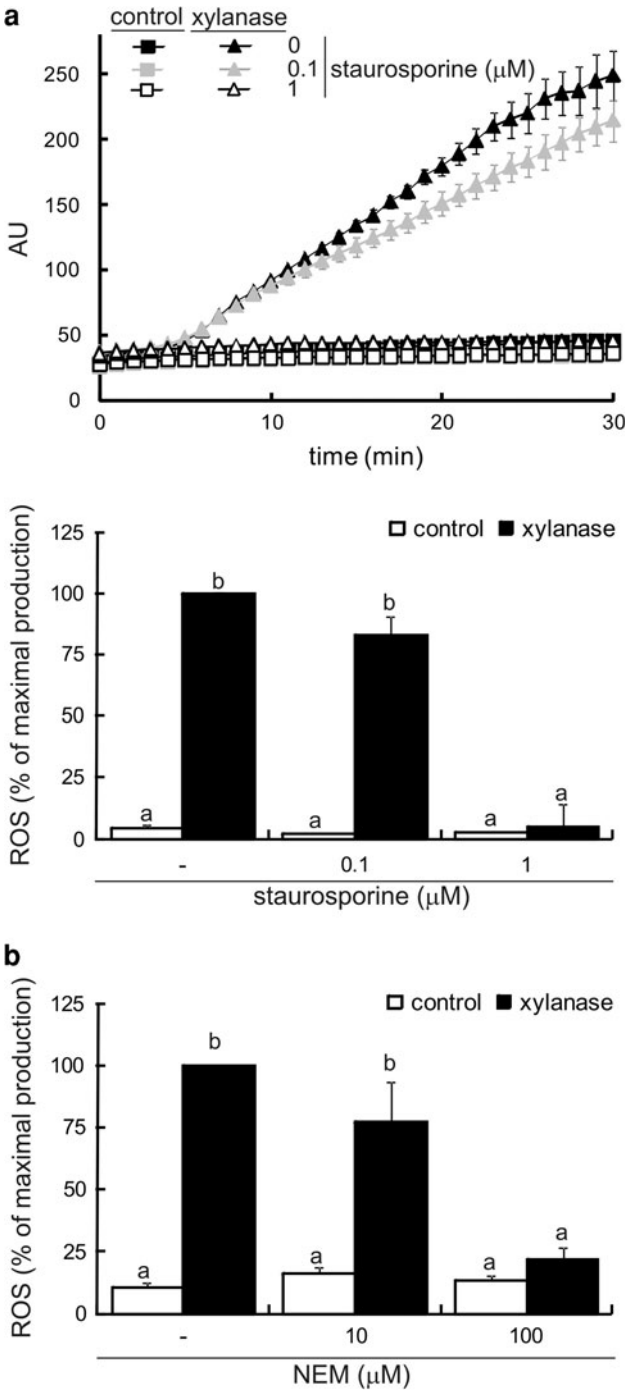
We then tested whether the xylanase signaling to ROS accumulation is dependent on protein S-nitrosylation events using the inhibitor NEM. The application of increasing doses of NEM reduced the increase of ROS triggered by xylanase to control values in tomato cell suspensions (Fig. 4b). We checked the effect of 0.025% (v/v) ethanol on ROS assays and found that treatment with the solvent resulted in a similar response compared to the control treatment (data not shown). Further experiments showed that ROS production, and not NO production, is abolished by treatment with *S*-methyl methanethiosulfonate, another cell permeable inhibitor of S-nitrosylation (data not shown).

Taken together, data presented in Fig. 4 support an involvement of both the protein phosphorylation and S-nitrosylation events in ROS formation during xylanase signaling in tomato cells.

Discussion

In the present study, we analyzed the participation of protein kinases, guanylate cyclase and the NO-mediated posttranslational modification S-nitrosylation in PA formation during xylanase signaling in tomato cells. Notwithstanding the limitations of the pharmacological approach used herein, data collectively indicate that upon xylanase perception the activation of a protein kinase pathway is required for NO formation and that protein S-nitrosylation-dependent mechanisms are involved in downstream signaling to PA and ROS.

Protein S-nitrosylation is highly recognized as an important posttranslational modification that mediates the diverse actions of NO (Stamler et al. 2001). Most of the information on S-nitrosylation has arisen from animal studies. Since NO is a lipophilic molecule, membrane proteins and lipoproteins are more exposed or susceptible to NO chemistry. The probability of a protein to be S-nitrosylated depends on the proximity of cysteine residues to the NO source, the redox state of the microambient and the identity of the flanking residues to the cysteine (Mannick and Schonhoff 2002). In plants, S-nitrosylation of proteins has attracted much less attention. However, there is substantial evidence regarding the in vivo effect of treatment with NEM in both the animal (Almanza et al. 2007; Jian et al. 2007; Pan et al. 2008; Tjong et al. 2008;



◀ **Fig. 4** Xylanase-mediated ROS accumulation is significantly inhibited by staurosporine and NEM. **a** Dose– and time–response curves of ROS production. Cells were incubated with CFM (control) or with xylanase in the presence or absence of different concentrations of staurosporine (0.1 and 1 μM). ROS level was determined over a 30 min period in a microwell plate fluorometer using the fluorescent probe H_2DCF-DA (4 μM). Fluorescence values are expressed as arbitrary unit (AU). *Upper panel* a representative graph is shown. *Error bars* represent SE of the three technical replicates within the experiment. *Lower panel* the gradient of ROS production from 5 to 15 min was calculated and expressed as percentage, 100% corresponding to the AU of xylanase-treated cells in absence of the inhibitor. *Error bars* represent SE of the means ($n = 4$). Means denoted by the same letter do not significantly differ at $P < 0.05$ according to One Way ANOVA. **b** Cells were incubated with CFM (control) or with xylanase in the presence or absence of 10 or 100 μM NEM. ROS level was determined and quantified as indicated in **a**. *Error bars* represent SE of the means ($n = 4$). Means denoted by the same letter do not significantly differ at $P < 0.05$ according to One Way ANOVA

Kawano et al. 2009) and plant cells. Extracellular application of NEM completely inhibited photosynthesis and respiration in *Beta vulgaris* leaf discs (Giaquinta 1976), stomatal opening in epidermal strips of *Pisum sativum* leaves (Rao and Anderson 1983), mitochondrial respiration in *P. sativum* (Chiandussi et al. 2002) and outward rectifying K^+ channel activity in *Vicia faba* stomatal guard cells (Sokolovski and Blatt 2004). Our study with the S-nitrosylation inhibitor NEM has indicated that the protein S-nitrosylation events are involved in PA and downstream ROS formation in xylanase-elicited tomato cells (Figs. 1, 4). It is therefore tempting to speculate that NO-dependent S-nitrosylation is the mechanism involved in the activation of PLC/DGK pathway during xylanase signaling. Nevertheless, we still do not know whether this is a direct S-nitrosylation of free cysteine thiol group(s) on tomato PLC(s) or DGK(s), or an indirect S-nitrosylation of protein(s) that activates the PLC/DGK pathway. Assuming that S-nitrosylation is the activation mechanism, we used NEM to explore the effect of this redox agent on the formation of PA triggered by the NO donor *S*-nitroso-*N*-acetylpenicillamine. We previously reported that high doses (0.5 and 1 mM) of this NO donor were effective in inducing PA production in the tomato cells (Laxalt et al. 2007). We found that exposure to up to 100 μM NEM had no effect on NO-mediated PA accumulation (data not shown). This unexpected result can be explained by analyzing the data in animals, where the applied doses of NEM ranged from 2 to 50 times higher the concentration of the NO donor applied (Almanza et al. 2007; Jian et al. 2007; Pan et al. 2008; Tjong et al. 2008; Kawano et al. 2009). Taking data into consideration, the application of at least 1 mM NEM could probably attenuate the positive effect of 0.5 mM *S*-nitroso-*N*-acetylpenicillamine on PA accumulation in tomato cell suspensions. We then studied the effect of 1 mM NEM in blocking the NO action and found that this concentration resulted toxic to tomato cells (data not shown). Hence, such experiments cannot be performed in our experimental system.

By the use of bioinformatics, we have previously found that the consensus S-nitrosylation motifs are not conserved in the primary sequences of plant PLCs and DGKs (Distéfano et al. 2010). Despite this fact, we cannot discard the possibility that these motifs could be present in their tertiary structures and thus be subject of S-nitrosylation.

Recently, data from the animal field have indicated that the activation mechanism of three human PLCs involves alkylation of several thiol groups of cysteines (Klein et al. 2011). In addition, it has been reported that H_2O_2 generates cytosolic Ca^{2+} oscillations by activating human PLCγ1 through sulfhydryl oxidation-dependent mechanisms (Hong et al. 2006). It would be interesting to analyze if plant PLCs or DGKs can suffer posttranslational modifications on cysteine residues and to test the effect of these modifications on their enzymatic activity.

It is well documented that the reversible protein phosphorylation is a key biological process for regulating pathogen-induced defense responses in plants. In this report, the general protein kinase inhibitor staurosporine abolished xylanase-induced NO, and downstream PA and ROS production (Figs. 2, 3, 4). This suggests that protein phosphorylation events are required for NO formation upon xylanase recognition in the tomato cell suspensions. Protein kinase inhibitors also suppressed cryptogein-induced NO production in tobacco cells (Lamotte et al. 2004) and NO accumulation in response to the potent elicitor from *Botrytis cinerea* BcPG1 in grapevine cells (Vandelle et al. 2006). Our observations are also in accordance with those obtained previously in which staurosporine was shown to block all the cryptogein-induced events monitored so far. Particularly, staurosporine inhibited Ca^{2+} influx and cytosolic Ca^{2+} elevation (Tavernier et al. 1995; Lecourieux et al. 2002). In addition, staurosporine inhibited the increase of cytosolic Ca^{2+} concentration, ROS production and MAPK activation triggered by *N*-acetylchitooligosaccharides (Kurusu et al. 2011). Altogether, these data indicate that protein phosphorylation is needed for a variety of early responses upon elicitor perception.

Since it has been demonstrated that the activation of different protein kinase families lies downstream of NO (Clarke et al. 2000; Klessig et al. 2000; Capone et al. 2004; Pagnussat et al. 2004; Sokolovski et al. 2005; Lanteri et al. 2006), we analyzed the possibility that another protein phosphorylation event could take place in transmitting the NO signals leading to the increase of PA. We then evaluated the level of PA in tomato cells treated with 0.5 mM of the NO donor *S*-nitroso-*N*-acetylpenicillamine in the presence or absence of different concentrations of the inhibitor staurosporine. As expected, treatment with NO generated a significant raise of PA in tomato cells (data not shown; Laxalt et al. 2007). However, the level of PA remained unaffected when staurosporine was tested in NO-treated cells (data not shown). Therefore, the activation of protein kinases is not a prerequisite for PA accumulation in response to NO. In conjunction, it can be concluded that protein kinases might be important components leading to NO formation and no further phosphorylation events are required for the signaling pathway downstream of NO that produces PA and ROS.

Reports from our and other labs have demonstrated an NO-dependent activation of CDPKs and MAPK cascades in tobacco (Klessig et al. 2000), *Arabidopsis* (Clarke et al. 2000; Capone et al. 2004), cucumber (Pagnussat et al. 2004; Lanteri et al. 2006) and *Vicia faba* (Sokolovski et al. 2005). Conversely, in this study, no significant effect was observed on xylanase-induced NO, PA and ROS production in treatments with the inhibitor of CDPKs TFP, the specific inhibitor of MAPKs SB202190 or the specific inhibitor of MAPK kinases PD098059 (Fig. 2 and data not shown). The reagent TFP is not specific for inhibiting CDPKs. It also affects the activity of other Ca^{2+}-binding proteins such as calmodulin and calcineurin B-like proteins (Anil and Rao 2000). Even though the limitations of pharmacology, we suggest that CDPK and MAPK signaling pathways are not involved in these responses triggered by xylanase in tomato cells. These data are consistent with a previous report that analyzed the effect of general and specific protein kinase inhibitors on UV-B-induced ROS production in *Catharanthus roseus* cell suspension cultures. Authors found that this response was suppressed by staurosporine but not by SB203580 and PD098059, two MAPK cascade inhibitors (Ramani and Chelliah 2007). This and other results suggest that a detailed analysis is needed to determine the involvement of a specific protein kinase family in a particular process.

As already described in the introduction, the activity of PLC and DGK enzymes is regulated by Ca^{2+} (Mueller-Roeber and Pical 2002). The synthesis of the second messenger cGMP is catalyzed via activation of the enzyme GC, and cGMP regulates cytosolic Ca^{2+} concentration via cADPR in both the animals and plants (Gow and Ischiropoulos 2001). Up to now, the involvement of cGMP in the regulation of the PLC/DGK pathway was not assessed. Evidence presented, herein, indicated that the blockage or administration of cGMP provoked no effect on PA accumulation in tomato cells (Figs. 1b, c). We also corroborated that the guanylate cyclase inhibitor LY83583 did not alter the increase of PA level in response to the NO donor *S*-nitroso-*N*-acetylpenicillamine (data not shown). These results suggest that, under our experimental conditions, a cGMP-mediated pathway is not involved in xylanase- or NO-induced PA formation. This is in agreement with the other reports that demonstrated that NO activates K^+ channels by S-nitrosylation but not via a cGMP-dependent signaling pathway (Tjong et al. 2008; Kawano et al. 2009). On the contrary, other reports showed that NO contribute to the regulation of cytosolic Ca^{2+} concentration via both a cGMP pathway and a direct action on Ca^{2+} channel proteins by S-nitrosylation reactions (Almanza et al. 2007; Jian et al. 2007).

It is important to elucidate exactly how NO, PA, and ROS are interrelated in the plant defense response. Data obtained from our lab have proposed that NO is upstream of PA, and that PA is upstream of ROS in xylanase-elicited tomato cells (Laxalt et al. 2007). We have also recently reported that, in chitosan-treated tomato cells, NO is required for PA generation via both the PLC/DGK and the PLD pathways, and that the inhibition of PLC/DGK-derived PA blocked chitosan-induced ROS production (Raho et al. 2011). Our present results are in favor that the sequence of events in xylanase signaling involves NO production, followed by PA production and finally ROS production.

Overall, our findings contribute to the identification of the mechanisms controlling the PA accumulation in the signaling pathway triggered by xylanase and NO, and highlight the remarkable complexity of plant–pathogen interactions. Further studies directed toward the protein S-nitrosylation will certainly improve our understanding of the role of this NO-dependent redox-based posttranslational modification on the regulation of the PLC/DGK pathway in the plants.

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