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Ozone and nitric oxide induce cGMP-dependent and -independent transcription of defence genes in tobacco

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Summary

- Here, we analyse the temporal signatures of ozone (O_3)-induced hydrogen peroxide (H_2O_2) and nitric oxide (NO) and the role of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) in transcriptional changes of genes diagnostic for biotic and abiotic stress responses.
- Within 90 min O_3 induced H_2O_2 and NO peaks and we demonstrate that NO donors cause rapid H_2O_2 accumulation in tobacco (*Nicotiana tabacum*) leaf. Ozone also causes highly significant, late (> 2 h) and sustained cGMP increases, suggesting that the second messenger may not be required in all early (< 2 h) responses to O_3, but is essential and sufficient for the induction of some O_3-dependent pathways.
- This hypothesis was tested resolving the time course of O_3-induced transcript accumulation of alternative oxidase (*AOX1a*), glutathione peroxidase (*GPX*), aminocyclopropancarboxylic acid synthase (*ACS2*) that is critical for the synthesis of ethylene, phenylalanine ammonia lyase (*PALa*) and the pathogenesis-related protein *PR1a*.
- The data show that early O_3 and NO caused transcriptional activation of the scavenger encoding proteins *AOX1a*, *GPX* and the induction of ethylene production through *ACS2* are cGMP independent. By contrast, the early response of *PALa* and the late response of *PR1a* show critical dependence on cGMP.

Introduction

An ever-increasing number of reports implicate the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) in many different physiological responses in higher plants (Newton & Smith, 2004; Meier & Gehring, 2006). They include the gating of K^+ channels (Hoshi, 1995; Leng *et al.*, 1999; Ma & Berkowitz, 2007), responses to NaCl, drought (Donaldson *et al.*, 2004) and biotic stresses (Durner *et al.*, 1998), stomatal guard cell regulation (Pharmawati *et al.*, 1998a; Cousson, 2001), gravitropic stimuli (Hu *et al.*, 2005) and the induction of gravity-directed cell polarity (Salmi *et al.*, 2007). In addition, cGMP has been implicated as second messenger in auxin (Pagnussat *et al.*, 2002), gibberellic acid (Penson *et al.*, 1996) and kinetin-dependent signalling (Pharmawati *et al.*, 1998b).

It is noteworthy that the time for cytosolic cGMP increases varies considerably from < 5 min in response to plant natriuretic peptides (Pharmawati *et al.*, 1998b) to 15 min in response to NaCl and osmotica (Donaldson *et al.*, 2004), 2 h after application of a nitric oxide (NO) donor (Durner *et al.*, 1998) and 5 h in response to a gravitropic stimulus (Hu *et al.*, 2005). This can be taken as an indication of the complex and diverse roles of cGMP as signalling molecule and might imply that cGMP has a role as a rapid transducer as well as in sustaining long-term adaptive responses.

Furthermore, a cGMP-dependent transcriptome has been obtained (Maathuis, 2006) and the presence of genes encoding monovalent cation transporters such as nonselective ion channels and cation/H^+ antiporters can provide a functional link to biotic and abiotic stress responses. Considering that so many highly diverse processes in higher plants are critically dependent on cGMP, it is somewhat surprising that only two *Arabidopsis* molecules have been reported that have guanylyl cyclase (GC) activity (Ludidi & Gehring, 2003; Kwezi *et al.*, 2007), particularly so, since the unicellular green alga *Chlamydomonas reinhardtii* contains > 100 nucleotide cyclases with 22 different domain combinations involving 13 different domain partners (Meier *et al.*, 2007) including the H-NOX domain

responsible for NO sensing in animal soluble GCs (Boon *et al.*, 2005).

While it remains unclear how NO is synthesized *in planta*, many biological processes including xylogenesis, programmed cell death, pathogen defence, flowering, stomatal closure, and gravitropism are NO dependent (for review see Shapiro, 2005; Neill *et al.*, 2007). In addition, we have demonstrated that NO plays a pivotal role in the response of tobacco plants to O_3 (Ederli *et al.*, 2006, 2008) and in particular, that NO is indispensable for the activation of the alternative oxidase gene (*AOX1a*) encoding a protein involved in the scavenging of mitochondrial reactive oxygen species (ROS). Nitric oxide may signal via multi-domain sensor molecules where an H-NOX domain (Boon *et al.*, 2005) combines with a GC domain, as in many annotated soluble GCs, thus linking NO to increasing GC activity. An alternative NO signalling pathway may involve reversible post-translational protein S-nitrosylation (Grennan, 2007). In summary, while the link between NO and cGMP dependent signalling is well established (Neill *et al.*, 2003; Prado *et al.*, 2004; Delledonne, 2005; Salmi *et al.*, 2007) the biochemical nature of the link remains to be discovered.

Here the aim was to characterize the role of cGMP in O_3- and NO-dependent transcriptional responses of genes with a role in plant defence and a particular emphasis is on resolving the temporal signature of the second messenger.

Materials and Methods

Plant material

The tobacco (*Nicotiana tabacum* L. cv. BelW3) seeds were kindly provided by J. Nicholson (NCSU Crop Science Department, Raleigh, NC, USA). Growth chamber conditions were: 14 h photoperiod, photosynthetic photon fluence rate (PPFR) of 120 μmol m^{-2} s^{-1}, day/night air temperature of 25°C/20°C, and relative humidity 60–75%. The fourth leaf from the apex of four treated and four untreated (controls) 12-wk-old plants were used in all experiments and the experiments were replicated four times.

O_3 treatment

Plants were exposed to 150 nl l^{-1} O_3 or to filtered air in Plexiglas chambers (0.32 m^3) under light with a PPFR of 400 μmol m^{-2} s^{-1} for up to 5 h (from 08 : 00 h to 13 : 00 h). The O_3 produced by UV irradiation (OEG50L lamp; Helios Italquartz s.r.l., Milan, Italy) was continuously monitored with a UV photometric O_3 analyser (Thermo Electron Corporation, Franklin, MA, USA). At different times during the 5 h of O_3 treatment and at 8 h and 24 h after starting ozone fumigation, the fourth leaves were sampled, frozen under liquid nitrogen and stored at −80°C until H_2O_2 determination and transcript analysis. For NO quantification fresh material was frozen under liquid N_2 and immediately analysed. To reduce NO and cGMP accumulation, before the onset of O_3 fumigation, the leaves were painted with 200 μM of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or 200 μM of the inhibitor of soluble GCs 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ).

Treatment of tobacco discs with chemicals

To evaluate the NO induced H_2O_2 accumulation, leaf discs (10 mm diameter) were floated in 5 mM sodium nitroprusside (SNP) or 1 U ml^{-1} of nitric oxide synthase (NOS) (Durner *et al.*, 1998). NOS (Calbiochem-Inalco, Milan, Italy) was obtained by expressing recombinant constitutive rat neuronal NOS in *Spodoptera frugiperda* cells using a baculovirus expression system. For enzymatic production of NO by NOS the following mixture was prepared: NOS (5 units) in 5 ml of 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), containing all cofactors and substrates of NOS (5 μM oxyhaemoglobin, 50 μM L-arginine, 100 μM $CaCl_2$, 20 μg ml^{-1} calmodulin, 120 μM NADPH, 12 μM tetrahydro-L-biopterin and 100 μM dithiothreitol (DTT)). Solutions of 10 mM K-phosphate buffer (pH 7.4) or substrate/cofactor mix lacking NOS were used as controls for SNP and NOS, respectively. The H_2O_2 was also quantified in leaf discs floated on NO-donors and 1 mM of the NO quencher cPTIO. After 1 h and 2 h incubation at 37°C under light (PPFR 400 μmol m^{-2} s^{-1}), leaf discs were sampled and H_2O_2 measured. To evaluate the H_2O_2-induced NO accumulation 100, 250 and 500 mM H_2O_2 or H_2O as control were injected with a syringe into intercellular space of tobacco leaf (Jabs *et al.*, 1996) and NO was quantified by haemoglobin assay in the tissue after 15, 30, 45 and 60 min. To evaluate transcript modulation AOX1a, PR1a, PALa, GPX and ACS2, leaf discs were floated for 1 h in the following solutions: 10 μM of the cGMP analogue 8-Br-cGMP, 5 mM SNP, 1 mM cPTIO, 200 μM ODQ and H_2O as control. After the incubation the leaf discs were transferred in H_2O and sampled for RNA extraction after 2, 3, 5 and 24 h. To test cGMP dependence of ethylene (ET) evolution after NO donor treatment, leaf discs were painted with 200 μM, 500 μM and 1000 μM ODQ alone or together with 5 mM SNP. As control, leaf discs were treated with H_2O. After 2 h ET accumulation was measured.

Quantification of NO by haemoglobin assay

The NO accumulation was assayed and calculated by following the conversion of oxyhaemoglobin (HbO_2) to methaemoglobin (MetHb) spectrophotometrically at 401 nm and 421 nm, using an extinction coefficient of 77 mM^{-1} cm^{-1} (A_{401} HbO_2, A_{421} MetHb) (Murphy & Noack, 1994). Oxyhaemoglobin was prepared as detailed previously (Clarke & Higgins, 2000). Leaf tissue (150 mg FW) was snap frozen and homogenized in a mortar with 100 mM K-phosphate buffer (pH 7.0) and 0.6% (w : v) insoluble polyvinylpolypyrrolidone. The extract

was clarified by adding powdered activated carbon and centrifuged at 11 000 ***g*** for 10 min at 4°C. The supernatant was filtered through a PTFE Millipore membrane (0.45 μm) and immediately assayed for NO. Five minutes before oxyhaemoglobin addition, samples were pretreated with catalase (100 U) and superoxide dismutase (100 U) to remove ROS. To evaluate the percentage of recovery of NO during extraction, 1 mM SNP, which releases 5 μM NO at room temperature and under light (PPFR 400 μmol m^{-2} s^{-1}), was added to leaf tissue and the measured recovery after Millipore filtration ranged from 68 to 75%.

Quantification of H_2O_2

Frozen leaves (200 mg FW) were ground to a powder under liquid N_2 and homogenized with 1 ml of 0.2 M $HClO_4$. The extract was kept on ice for 5 min and centrifuged at 10 000 ***g*** for 10 min at 4°C. The acidic supernatant was neutralized to pH 7.0–8.0 with 0.2 M NH_4OH pH 9.5 and centrifuged at 3000 ***g*** for 2 min to sediment the insoluble material. The recovered supernatant was passed through column of AG 1-X8 resin (Bio-Rad, Hercules, CA, USA) and was eluted with distilled water. The H_2O_2 concentrations were determined spectrophotometrically by xylenol orange assay, as reported previously (Pasqualini *et al.*, 2003). To evaluate the percentage of recovery of H_2O_2 during extraction and purification of samples, 2 μmol H_2O_2 were added to leaf tissue. Recovery after column elution ranged from 90 to 98%.

Cell viability

Cell death was evaluated by histochemical analysis using trypan blue. For trypan blue staining, samples were covered with an alcoholic lactophenol trypan blue mixture (30 ml of ethanol, 10 g of phenol, 10 ml of water, 10 ml of glycerol, 10 ml of lactic acid and 10 mg of trypan blue), placed in a boiling water bath for 2 min, left at room temperature for 1 h, then transferred into a chloral hydrate solution (2.5 g ml^{-1}) and boiled for 20 min to destain. After multiple changes of chloral hydrate solution to reduce the background, samples were equilibrated with 50% glycerol, mounted and observed with a stereomicroscope.

Determination of ET

The determination of ET from leaf segments was performed as detailed previously (Ederli *et al.*, 2006).

Quantification of cGMP

Frozen tissue (200 mg) was ground to a fine powder, added to 2 ml of ice-cold 6% trichloroacetic acid and the homogenate centrifuged at 4000 ***g*** for 15 min. The aqueous extract was extracted four times in five volumes of diethyl ether, dried under vacuum at 25°C overnight and stored at −70°C. Cyclic GMP was determined according to the manufacturer's instructions for the cGMP Assay System (Amersham Bioscience, Piscataway, NJ, USA).

Cyanide analysis

Since is well-documented that both NO and cyanide (CN) are released during the decomposition of SNP (Bethke *et al.*, 2006) and cyanide has been reported to transcriptionally activate *AOX* gene in tobacco (Sabar *et al.*, 2000), we measured CN developed from 5 mM SNP solution with a cyanide detection kit (Model CYN-3; Hach Company, Loveland, CO, USA). Cyanide was undetectable after 1 h, which is the incubation time used to induce transcription in leaf discs (see the Supporting Information, Fig. S1). Significant CN concentrations were detected only after 5 h incubation and peaked at 24 h (Fig. S1).

Isolation and analysis of RNA

Total RNA was extracted from frozen, homogenized leaf tissue (100–150 mg FW) using NucleoSpin RNA Plant (Macherey-Nagel, Düren, Germany). One microgram of total RNA was reverse transcribed for 1 h at 42°C with 200 units of SuperscriptII RT (Invitrogen, Carlsbad, CA, USA) in 10 mM DTT, 0.4 mM of each dNTP and 0.5 μg oligo $dT_{12–18}$ primer (Invitrogen, Carlsbad, CA). The cDNA was used for polymerase chain reaction (PCR) with 1 unit *Taq* polymerase in 0.2 mM of each dNTP and 10 μM of the respective primers (see Table S1). The amplification steps were: initial heating at 94°C for 75 s, denaturation at 94°C for 5 s, annealing for 20 s, 72°C for 45 s and a final extension at 72°C for 7 min. The authenticity of the PCR products was checked by two directional sequencing using an ABI Prism 310 Genetic Analyzer (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). For quantification, filters were scanned and band intensities were determined with image analysis software.

Results

To test if O_3 triggers an oxidative burst, we quantified intracellular H_2O_2 accumulation after fumigation of whole plants. A highly significant transient increase of H_2O_2 to > 2.0 μmol g^{-1} FW was registered after 1.5 h of O_3 fumigation. Subsequently, the levels declined but remain significantly higher than the control for up to 5 h (Fig. 1a). After 5 h of treatment the intracellular H_2O_2 had returned to control levels of 0.53 μmol g^{-1} FW.

Simultaneous spectrophotometric monitoring of NO accumulation revealed that it significantly increased after 1 h, with a highly significant peak at 1.5 h where the level increased > 7 times from 0.13 to 0.91 nmol g^{-1} FW min^{-1}. At ≥ 3 h NO concentrations reverted to pretreatment levels (Fig. 1b). There was a temporal overlap between the

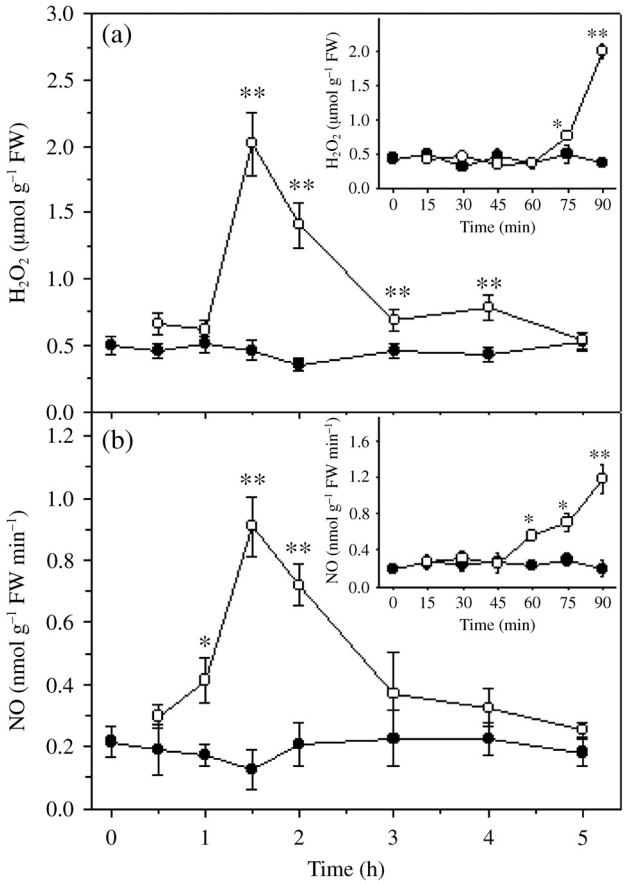

Fig. 1 Hydrogen peroxide (H_2O_2) and nitric oxide (NO) accumulation in ozonated leaves of tobacco (*Nicotiana tabacum*). H_2O_2 (a) and NO (b) accumulation in leaf extracts from control (closed circles) and fumigated (open circles) plants. Plants were fumigated with 150 nl l^{-1} O_3 for 5 h or were maintained in filtered air for the same period. Insets: the average ± SE of two experiments in which the sampling was performed at 15 min to best resolve the time-course of H_2O_2 and NO accumulation. Significance of variance for one-way ANOVA is indicated: *, $P \leq 0.05$; **, $P \leq 0.01$.

O_3-dependent H_2O_2 and the NO response signature with the latter showing an earlier onset and reversion to the base line.

To determine whether O_3 induces H_2O_2 and NO transients independently rather than being causally linked, we used a foliar disc assay. First, to test if NO can induce H_2O_2 increases, foliar discs were exposed to 5 mM SNP or recombinant rat neuronal NOS synthase. The result showed that both can induce highly significant H_2O_2 accumulation in leaf discs (Fig. 2a) and while 5 mM SNP triggers a larger accumulation of H_2O_2, the response to 1 U ml^{-1} NOS was more rapid. Confirmation that H_2O_2 accumulation was caused by NO came from the demonstration that the NO scavenger cPTIO suppressed both SNP- and NOS-induced H_2O_2 accumulation (Fig. 2a). Second, we tested if H_2O_2 could induce NO accumulation. Since H_2O_2 is rapidly degraded by endogenous scavengers, we quantified tissue H_2O_2 levels after 15 min treatment with different concentrations of H_2O_2 (Fig. 2b) and noted that a concentration as low as 100 mM H_2O_2 induced highly significant increases. However, H_2O_2 concentrations as high as 500 mM failed to significantly alter tissue NO levels (Fig. 2c) indicating that, in this experimental system, H_2O_2 does not induce NO accumulation in tobacco leaf. In order to exclude the possibility that the elevated H_2O_2 and SNP concentrations used to induce NO accumulation caused cell death and suppression of enzymatic NO production, we assayed cell viability with trypan blue and found no evidence that suggested that viability was affected (Fig. S2). Conversely, 5 h of O_3 fumigation induced cell death which was not uniformly distributed throughout the leaf, but appeared as spots (Fig. S2).

To elucidate whether the second messenger cGMP has a role in the O_3-induced responses we measured cellular cGMP levels during fumigation. A highly significant rise of cGMP level was detected after 3 h and significantly elevated levels persisted over the next 2 h (Fig. 3a). Moreover, the guanylate cyclase inhibitor ODQ at 200 μM suppressed the O_3-induced

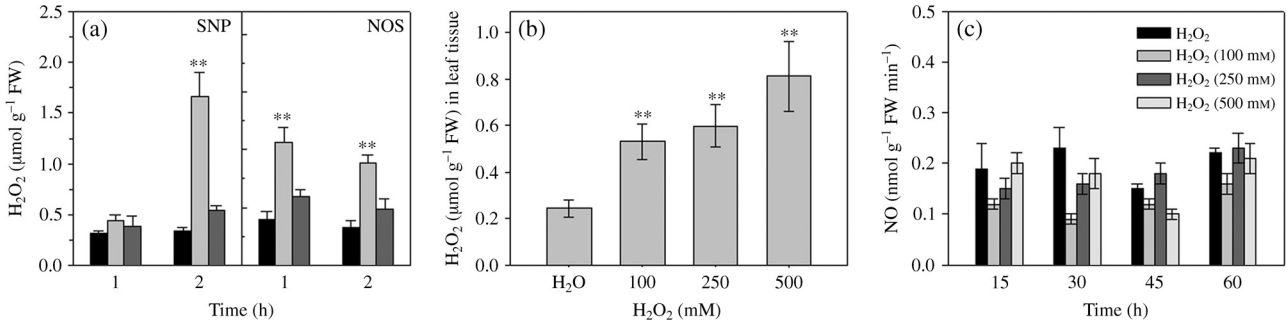

Fig. 2 Hydrogen peroxide (H_2O_2) and nitric oxide (NO) accumulation in leaf tobacco (*Nicotiana tabacum*) discs. (a) H_2O_2 concentration measured in tobacco leaf discs floated for 1 h and 2 h with the NO donors 5 mM sodium nitroprusside (SNP) and 1 U ml^{-1} nitric oxide synthase (NOS, lighter tinted bars), with 10 mM K-phosphate buffer (pH 7.4) or substrate/cofactor mix lacking NOS, respectively, as control for SNP and NOS (closed bars) or with 5 mM SNP + 1 mM 2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or 1 U ml^{-1} NOS + 1 mM cPTIO (darker tinted bars). (b) H_2O_2 concentration measured in leaf discs after 15 min from the infiltration with 100, 250 and 500 mM H_2O_2 or H_2O as control. (c) The NO content measured in leaf discs by haemoglobin assay at different time-points after injection with different H_2O_2 concentration. The data are the mean ± SE from four independent experiments. Significance of variance for one-way ANOVA: **, $P \leq 0.01$.

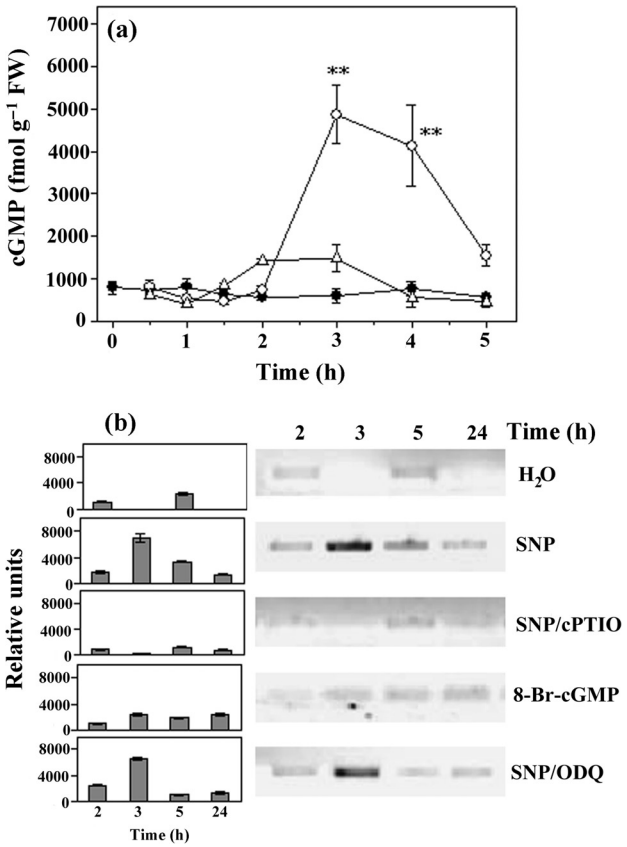

Fig. 3 Cyclic GMP generation and *AOX1a* response. (a) Cyclic GMP levels measured at different time points in leaves of tobacco (*Nicotiana tabacum*) plants fumigated with 150 nl l^{-1} O_3 (open circles), maintained in filtered air for the same period (closed circles) or pretreated with 200 μM 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) before the ozone fumigation (triangles). The data are the means ± SE from four independent experiments. Significance of variance for one-way ANOVA: *, $P \leq 0.05$; **, $P \leq 0.01$.
(b) Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for *AOX1a* in response to treatment with 5 mM sodium nitroprusside (SNP), 5 mM SNP plus 1 mM 2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 10 μM 8-Br-cGMP, 5 mM SNP plus 200 μM ODQ or H_2O as control. Leaf discs were floated for 1 h with chemicals and then transferred in H_2O. At the indicated times total RNA was isolated from leaf tissue. Total RNA was reverse transcribed and amplified by RT-PCR. Semi-quantification of mRNA levels loaded in each lane was performed by co-amplification and normalization with an internal standard (*actin*). Beside each blot, a graph with relative intensities of the signals ± SE is shown.

cGMP accumulation (Fig. 3a). It is noteworthy that the cGMP elevation follows both the O_3-induced H_2O_2 and NO peaks. Given that NO is an essential signal molecule for the activation of the mitochondrial *AOX1a* (Ederli *et al.*, 2006, 2008), we were interested to resolve whether cGMP has a role in the transcriptional activation of this gene even though significant NO dependent cGMP increases above basal levels were registered only after the onset of O_3-dependent *AOX1a* transcript accumulation (≥ 1.5 h) (Ederli *et al.*, 2006).

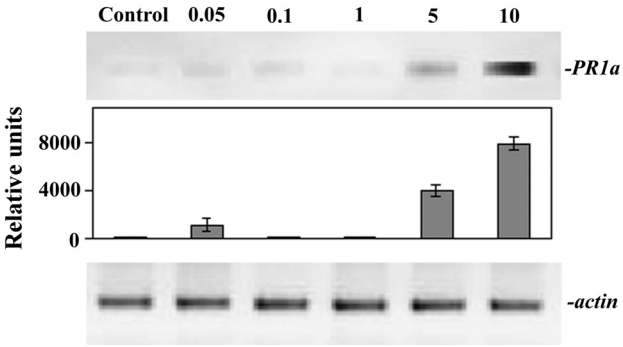

Fig. 4 Cyclic GMP dependent *PR1a* transcription. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for *PR1a* in response to treatment with different concentrations (from 0.05 to 10 μM) of 8-Br-cGMP or with H_2O as control. Tobacco (*Nicotiana tabacum*) leaf discs were floated with 8-Br-cGMP for 24 h and after this time total RNA was isolated from tissue leaf. Total RNA was reverse transcribed and amplified by RT-PCR. Semi-quantification of mRNA levels loaded in each lane was performed by co-amplification and normalization with an internal standard (*actin*). A graph with relative intensities of the signals ± SE is shown below the blot.

The cell-permeant analogue 8-Br-cGMP was used to test whether cGMP can activate transcription of *AOX1a* (Fig. 3b). Despite the fact that treatment with 1 μM 8-Br-cGMP delivers 8233.29 fmol 8-Br-cGMP g^{-1} FW to leaf tissue, no transcriptional induction of *AOX1a* was detected, while the NO donor SNP induced transcript accumulation after 3 h. Furthermore, the effect of the NO donor was completely suppressed in the presence of the NO scavenger cPTIO, while the presence of ODQ, a selective inhibitor of NO-sensitive soluble GCs, did not affect NO-dependent transcription. It was thus concluded that the NO effect on *AOX1a* does not depend on a cGMP transient.

Given that cGMP has been established as a key signal component in the NO-dependent *PR1a* induction (Delledonne *et al.*, 1998; Durner *et al.*, 1998), we tested different 8-Br-cGMP concentrations for inducibility in our system and found that 10 μM 8-Br-cGMP causes the highest induction (Fig. 4). Subsequently, other defence genes were tested for cGMP-dependent transcriptional activation. They included the ROS detoxification enzyme glutathione peroxidase encoding gene (*GPX*), the aminocyclopropancarboxylic acid synthase gene involved in the synthesis of ethylene (*ACS2*), a gene encoding a protein with a role in stress-induced secondary metabolite synthesis, phenylalanine ammonia lyase (*PALa*) and the pathogenesis-related protein 1a (*PR1a*) (Fig. 5). Induction was monitored over 24 h at 2, 3, 5 and 24 h and *GPX* and *ACS2* (Fig. 5a,b), much like *AOX1a*, were NO inducible but did not require cGMP increases. *PALa* and *PR1a* are also NO inducible but induction is critically cGMP dependent (Fig. 5c,d). The second messenger cGMP is sufficient to induce *PALa* and *PR1a* while induction is almost completely suppressed in the presence of the soluble GC inhibitor ODQ (Fig. 5c,d), suggesting a critical role of cGMP in the signalling pathway. The temporal induction profiles of *PALa* and *PR1a* are distinctly

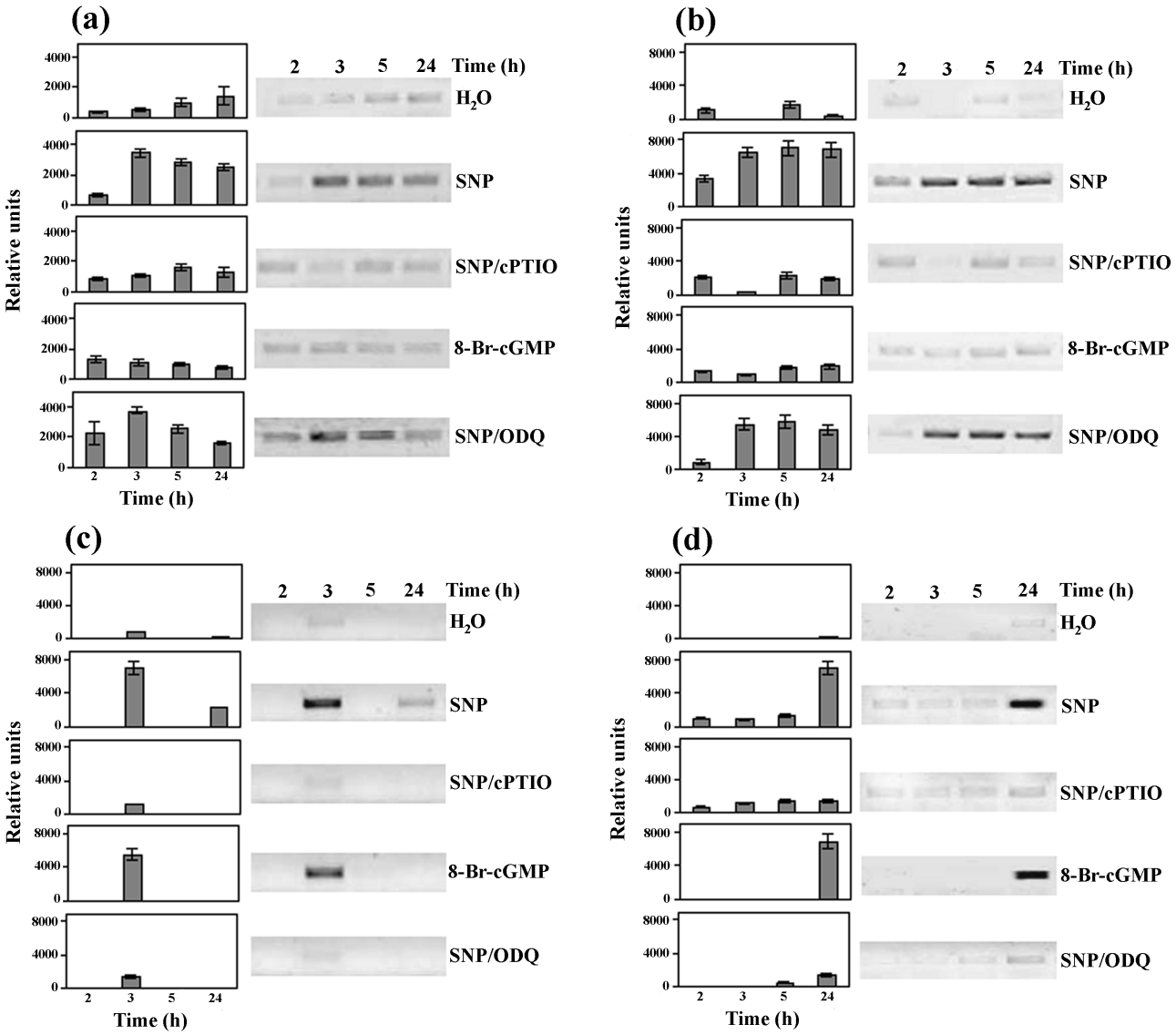

Fig. 5 Nitric oxide (NO)-dependent transcriptional responses. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for *GPX* (a), *ACS2* (b), *PALa* (c) and *PR1a* (d) in response to treatment with 5 mM sodium nitroprusside (SNP), 5 mM SNP plus 1 mM 2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 10 μM 8-Br-cGMP, 5 mM SNP plus 200 μM 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) or H_2O as control. Tobacco (*Nicotiana tabacum*) leaf discs were floated for 1 h with chemicals and then transferred in H_2O. At the indicated times, total RNA was isolated from tissue leaf. Total RNA was reverse transcribed and amplified by RT-PCR. Semi-quantification of mRNA levels loaded in each lane was performed by co-amplification and normalization with an internal standard (*actin*). A graph with relative intensities of the signals ± SE is shown beside each blot.

different, the former shows a transient peak at 3 h, while the latter is elevated above control levels at 24 h only, both in response to NO and 8-Br-cGMP. Since the NO-dependent cGMP levels occur at 3 h, direct induction can only be postulated for *PALa*.

Transcription of the *AOX1a*, *GPX*, *ACS2*, *PALa* and *PR1a* genes are induced by O_3 (Fig. 6). Since *AOX1a* is NO induced (Fig. 3b) and NO accumulates in fumigated leaves (Fig. 1b), we tested whether O_3-induced *AOX1a* upregulation can be prevented in ozonated plants pretreated with the NO quencher cPTIO. The result (Fig. 6b) indicated that *AOX1a* mRNA accumulation was completely abolished in NO-suppressed (+cPTIO) plants, thus suggesting that NO is indispensable for *AOX1a* induction. Similar to *AOX1a*, induction of the other NO-responsive genes (e.g. *GPX*, *ACS2*, *PALa* and *PR1a*) was also suppressed when the plants were pre-treated with cPTIO (data not shown).

In summary, the genes that encode proteins with a role in scavenging or ET-dependent signalling (*AOX1a*, *GPX* and *ACS2*) show cGMP independent induction, while those encoding proteins with a role in defence-related secondary metabolism (*PALa* and *PR1a*) appear to be cGMP dependent. We also

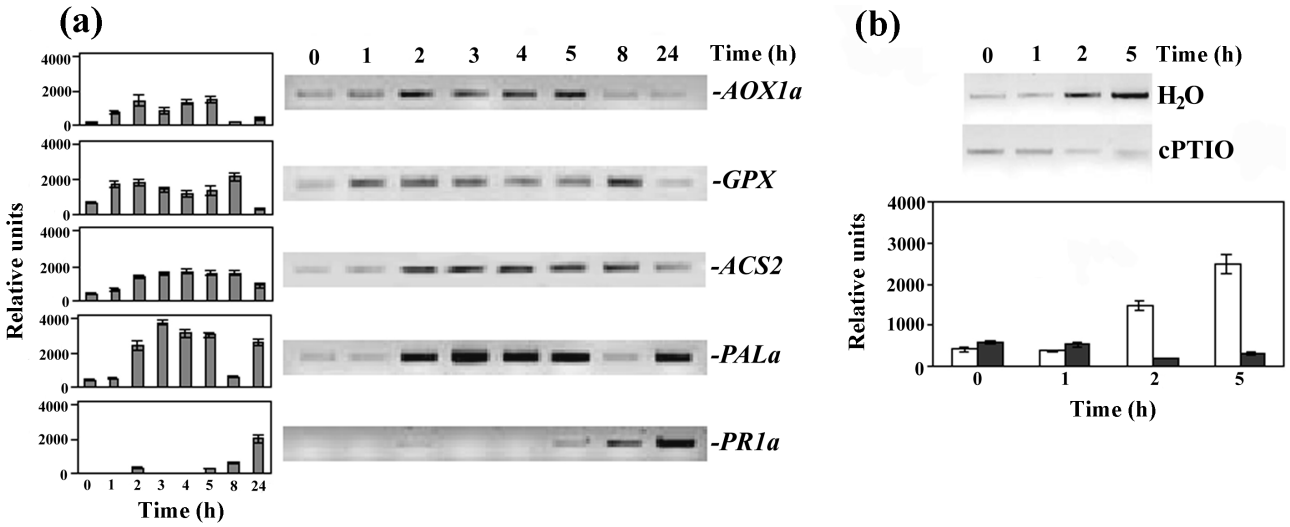

Fig. 6 Ozone (O_3) dependent transcriptional responses. (a) Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for *AOX1a*, *GPX*, *ACS2*, *PALa*, and *PR1a* genes from O_3-treated tobacco (*Nicotiana tabacum*) plants (150 nl l^{-1} O_3 for 5 h). Plants were sampled before O_3 fumigation (0 h) and after 1, 2, 3, 4, 5, 8 and 24 h from the start of fumigation. Total RNA was reverse transcribed and amplified by RT-PCR. Semi-quantification of mRNA levels loaded in each lane was performed by co-amplification and normalization with an internal standard (*actin*). A graph with relative intensities of the signals ± SE is shown beside each blot. (b) Changes in levels of the AOX1a transcript from O_3-fumigated plants after treatment with the NO scavenger 2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, closed bars). Leaves were brushed with 200 μM cPTIO 1.5 h before fumigation. This treatment was repeated three times during the fumigation. Control plants were brushed with distilled water (open bars). Sampling for mRNA analysis was done before and after 1, 2 and 5 h after the start of fumigation. Total RNA was reverse transcribed and amplified by RT-PCR. A graph with relative intensities of the signals ± SE is shown under the blots.

noted that *PALa* increases are observed 2 h after the onset of O_3 fumigation at a time when H_2O_2 levels have peaked (Fig. 1a) but before cGMP levels are detectable above control (Fig. 3a) and that the induction shows biphasic behaviour within the first 24 h. The first transient between 2 h and 6 h was previously reported in BelW3 (Ogawa *et al.*, 2005).

In both whole plants and leaf discs NO induces ET synthesis via the activation of *ACS2* (Ederli *et al.*, 2006). This NO-mediated effect on ET synthesis appears not to be directly dependent on cGMP (Fig. 5b). To further clarify the relationship between cGMP and ET, we floated foliar discs in 8-Br-cGMP and measured ET accumulation (Fig. 7a). The results demonstrates that no ET evolution occurs in the presence of 10 μM 8-Br-cGMP alone over the 5 h experimental time, indicating that cGMP is not sufficient for ET synthesis. However, when we treated tobacco leaf discs with SNP in the presence of ODQ we found that the NO-induced ET evolution was drastically inhibited in an ODQ concentration-dependent manner (Fig. 7b). The same result was obtained when the plants were pretreated with ODQ before the ozone fumigation. In this case too, no ET evolution was detected in fumigated plants, despite a peak of ET after 5 h of O_3 fumigation in plant without ODQ (data not shown).

Discussion

The second messenger cGMP is increasingly recognized as an important signalling molecule that is involved in a diverse number of physiological responses in plants. Similar to the second messenger Ca^{2+}, we are starting to see different signatures associated with response types, ranging from < 1 min (Donaldson *et al.*, 2004), to rapid transients with a peak at < 5 min after the stimulus (Pharmawati *et al.*, 1998b), to the delayed more sustained response with significant increases occurring after 2 h and remaining elevated after 3 h (Durner *et al.*, 1998) post NOS stimulus. The two different response types are indicative for an involvement of cGMP in both rapid signal transduction and modulation of ion channels (Hoshi, 1995; Leng *et al.*, 1999) and late long-term adaptive responses sustaining changes in the transcriptome (Maathuis, 2006) and the proteome.

Given that in plants cGMP has been reported to increase in response to NO (Durner *et al.*, 1998), and that O_3 induces increases in NO levels, we tested whether O_3 induction was also causing downstream cGMP elevations. Since O_3-dependent NO elevations occur after 1 h, and considering previously reported NO dependent cGMP transients (Durner *et al.*, 1998), one might expect that subsequent cGMP increases would occur 2 h later and exactly mirror the timing of previous studies. Consistent with these expectations, we did not observe a short early cGMP transient; however a highly significant cGMP increase was detected after 3 h of O_3 treatment which corresponds to the delayed, late and sustained second type of response (Fig. 3a).

This finding raises a number of major questions such as: What is the cascade of events leading to O_3-induced elevated cGMP levels? How is cGMP generated? and What are the

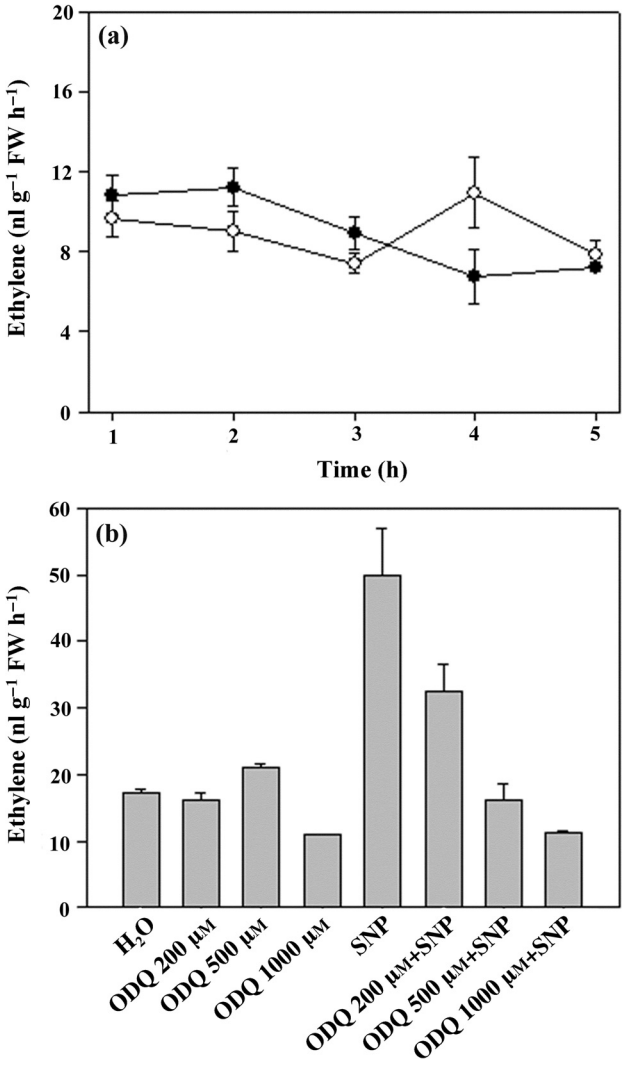

Fig. 7 Cyclic GMP dependence of ethylene (ET) evolution. (a) Ethylene emission from pieces of tobacco (*Nicotiana tabacum*) leaf after infiltration for the time indicated with 10 µM 8-Br-cGMP (open circles) or with H_2O (closed circles) as control. The data are the mean ± SE from four independent experiments. (b) Ethylene emission by leaf pieces after 2 h from application of 5 mM SNP, 200, 500 and 1000 µM 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) or ODQ (200, 500 and 1000 µM) + 5 mM sodium nitroprusside (SNP). The data are the means ± SE from two independent experiments.

consequent cGMP dependent responses? The answer to the first question is complicated by the fact that O_3 triggers transient increases in both cellular levels of H_2O_2 and NO, both peaking at 90 min with the onset of significant changes in NO levels occurring 15 min before the changes in cellular H_2O_2 (Fig. 1a,b). The fact that the H_2O_2 transients can also be induced by the NO donors (Fig. 2a) would suggest that O_3 can activate NO-dependent and -independent pathways, both contributing to the generation of H_2O_2 possibly leading to potentiation of the response. We have also ascertained that NO increases are independent of H_2O_2 levels and likely occur upstream of H_2O_2 since H_2O_2 increases do not affect NO levels (Fig. 2c). It is noteworthy that this sequence – NO increases preceding H_2O_2 increases – has also been reported in response to an avirulent *Pseudomonas syringae* strain in *Arabidopsis* suspension culture cells (Clarke *et al.*, 2000). However, our results are not in agreement with Zhang *et al.* (2007) who show that exogenous application of H_2O_2 to maize leaves induced NO generation. The discrepancy between the studies may be related to the different methods of NO detection employed. Zhang *et al.* (2007) reported that NO detection after H_2O_2 application was performed using the NO-sensitive dye DAF-2DA, and this is likely to lead to NO overestimation when oxidant and NO are cosynthesized (Jourd'heuil, 2002), while in our experiments NO was detected spectrophotometrically with a haemoglobin assay.

To assess whether transcriptional changes resulting from O_3 treatment of our target genes – *AOX1a*, *GPX*, *ACS2*, *PALa* and *PR1a* – were NO and/or cGMP dependent, we monitored their transcription in the presence of various combinations of the following agonists and antagonists: the NO donor SNP, the NO scavenger cPTIO, the cell permeant cGMP analogue 8-Br-cGMP and the soluble GC inhibitor ODQ (Figs 3b, 5).

In the case of *AOX1a*, we observed that NO-dependent transcription remained unaffected by the inhibition of soluble GCs and the gene was not induced by the cell-permeant cGMP analogue. It is thus concluded that cGMP has no direct role in the transcriptional regulation of *AOX1a* (Fig. 3b). The response to NO shows a distinct transient with transcripts accumulating above control levels only 3 h after SNP application.

The responses of *GPX* and *ACS2* are similar in that they are NO dependent but cGMP independent; however, in SNP-treated leaf discs the transcript levels stayed higher than the control for the duration of the monitored period of 24 h (Fig. 5a,b). Taken together, two genes that encode proteins with a role in alleviating oxidative stress – *AOX1a* and *GPX* – are both induced rapidly and cGMP independently. AOX1a directly prevents the mitochondrial oxidative stress while GPX may be critical for the oxidative damage protection of the chloroplast in particular. The third gene, which is not directly dependent on cGMP, is *ACS2*. Aminocyclopropancarboxylic acid synthase has a role in ET biosynthesis, and it is known to increase in response to O_3 in tobacco (Ogawa *et al.*, 2005; Ederli *et al.*, 2006), *Arabidopsis* (Rao *et al.*, 2002) and citrus plants (Iglesias *et al.*, 2006). However, when we treated tobacco leaf discs with the NO donor SNP and the soluble GC inhibitor ODQ it was found that NO-induced ET evolution was suppressed (Fig. 7b), suggesting that transcription of *ACS2* is not dependent on cGMP but downstream processes (e.g. post-transcriptional processes) involved in ET synthesis may critically depend on cGMP.

By contrast, NO-induced *PALa* and *PR1a* transcript accumulation were both cGMP dependent, with the former showing a transient increase after 3 h while the latter showed

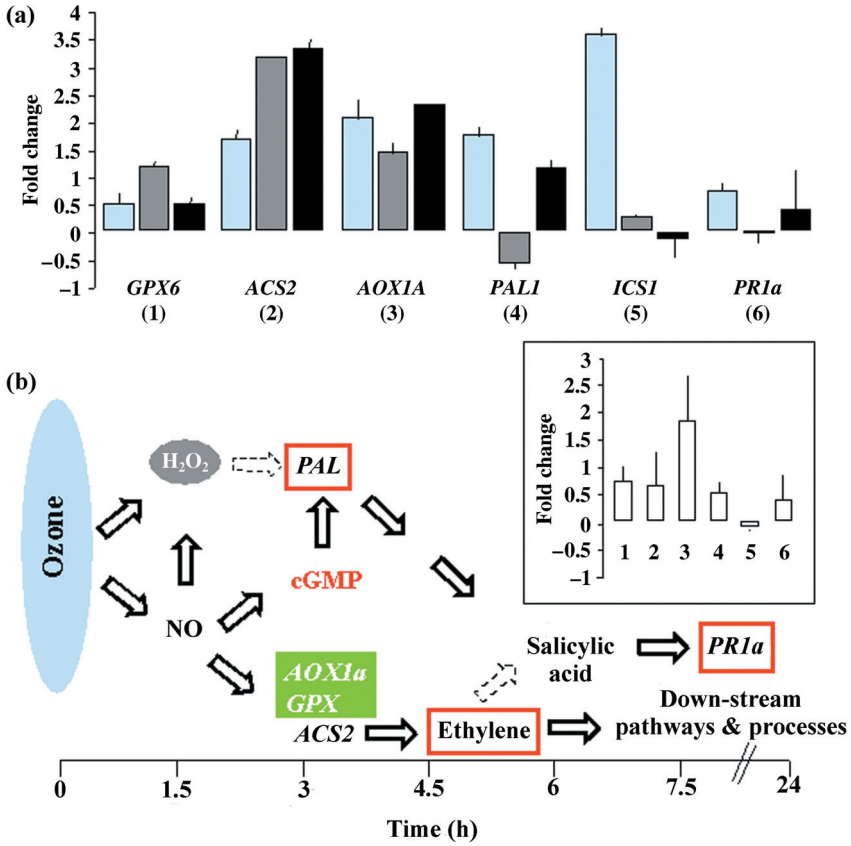

Fig. 8 Defence gene induction in *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*). (a) The results are the fold changes (log_2) in expression of *GPX6* (At4g11600), *ACS2* (At4g11280), *AOX1A* (At3g22370), *PAL1* (At2g37040), *ICS1* (AT1g74710) and *PR1a* (At2g14580) in *A. thaliana* in response to 6 h of O_3 (500 nl l^{-1}; light tinted bars) (NASCArray, ID O_3 –26, $n = 3$), 1 h of 20 mM H_2O_2 treatment (dark tinted bars; $n = 3$) or 3 h of 10 mM H_2O_2 treatment (closed bars; $n = 3$) (E-GEOD –3709). Error bars represent standard errors of the mean. (b) Model of O_3-induced responses in BelW3 tobacco plants. The solid box indicates genes directly involved in early oxidation protection. The genes that can be induced by cGMP and the compounds for which the synthesis is cGMP-dependent are in an empty box. Dotted arrows are experimentally unconfirmed links. Inset: fold change (log_2) in expression of the *Arabidopsis GPX6* (1), *ACS2* (2), *AOX1A* (3), *PAL1* (4), *ICS1* (5) and *PR1a* (6) in response to 3 h treatment with 10 μM salicylic acid (SA) (TAIR ATGenExpress, salicylic acid-ME00364; $n = 2$).

a delayed response at 24 h (Fig. 5c,d). We report an early O_3 activation of *PALa*, starting after 2 h of fumigation with peaks between 3 and 5 h, and a late activation after 24 h. This early O_3-dependent onset is consistent with salicylic acid (SA) accumulation after 5–7 h fumigation (Pasqualini *et al.*, 2002) since in tobacco PAL is a precursor of SA, whereas in *Arabidopsis thaliana* isochorismate synthase (*ICS*) is responsible for SA synthesis (Wildermuth *et al.*, 2001). The secondary late activation of *PALa* at 24 h after the onset of O_3 fumigation or at 96 h after challenge with the virulent strain of *P. syringae* was also observed in *Arabidopsis* (data not shown). However, the biological role remains unresolved. It is conceivable that while the early induction is responsible for SA synthesis, late induction could be the consequence of necrotic processes (Kangasjarvi *et al.*, 1994). Incidentally, the late and second *PALa* induction in response to a challenge with avirulent pathogens has been reported previously in *Arabidopsis* (Zeier *et al.*, 2004), implying that induction of PAL by O_3 may be mechanistically similar to the pathogen defence responses. We also noted that *PALa* induction initiates before the massive cGMP accumulation and it is conceivable that it may be caused by H_2O_2 (Desikan *et al.*, 1998).

We have also analysed *Arabidopsis* microarray data of the above genes including isochorismate synthase (*ICS1*) in response to 1 h and 3 h H_2O_2 exposure and 6 h O_3 exposure (Fig. 8a). In the case of H_2O_2, the most pronounced response is seen by *ACS2* linking H_2O_2 to the synthesis and responses to ET. The anti-oxidation protecting *AOX1A* and *GPX6* are also induced whereas *PAL1* and *PR1a* are upregulated after 3 h only. Ozone at 6 h causes transcript accumulation of all genes and notably *ICS1* responsible for SA synthesis in *Arabidopsis*. The fact that SA may slightly reduce *ICS1* expression (Fig. 8b, inset) suggests a negative feedback regulation of SA production, while the stimulation of *GPX6*, *ACS2*, *AOX1A*, *PAL1* and *PR1a* by SA is indicative for a role in a more sustained response for the protection of organelles as well as ET synthesis and consequent downstream responses. Inspecting the *Arabidopsis* cGMP-dependent transcriptome (supplementary data (Maathuis, 2006)) we noted that cGMP leads to an increase in *ICS* transcript levels both at 2 h and 5 h thus making the response similar to that in the tobacco model where we propose that cGMP can cause SA increases through the transcriptional activation of *PAL*.

While cGMP is accepted as an important second messenger in higher plants, the search for molecules that can catalyse the synthesis of cGMP from GTP in higher plants is complicated by the fact that BLAST searches with GC domains from either higher or lower eukaryotes do not yield any positive hits, suggesting that higher plants have evolved unique GCs where only the catalytic centre (Liu *et al.*, 1997) contains any degree of conservation (Ludidi & Gehring, 2003).

We have learned from animal GCs that essentially two distinctly different GC domain architectures exist – that of soluble

and that of particulate GCs. The former typically have a highly conserved NO-binding site consisting of an H-NOX domain and play a key role in NO sensing and signal transduction; the latter serve as transmembrane receptors where the GC domain located next to a kinase domain is in the cytosol (Meier *et al.*, 2007). Since we report that NO-dependent induction of *PALa* and *PR1a* are inhibited by ODQ (Fig. 5c,d), a selective inhibitor of NO-sensitive soluble GCs, we might expect that the GCs involved are activated by NO and possibly contain an H-NOX domain. Such dual domain enzymes common in animals are also annotated in *Chlamydomonas reinhardti* but have not been reported in higher plants (Meier *et al.*, 2007). There are, however, proteins in higher plants that contain the catalytic core of the H-NOX domain (Hx{12}Px{14,16}YxSxR) and it remains to be seen if any of them are directly or indirectly associated with NO binding and the generation of cGMP.

Accession number

Sequence data from this article can be found in the NCBI/GenBank data libraries under the following accession numbers: S71335 (*AOX1a*), AJ005002 (*ACS2*), AB008199 (*PALa*), AB041518 (*GPX*), X06361 (*PR1a*) and AF15640 (*actin*).

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References

Bethke PC, Libourel IGL, Jones RL. 2006. Nitric oxide reduces seed dormancy in *Arabidopsis*. *Journal of Experimental Botany* **57**: 517–526.

Boon E, Huang S, Marletta M. 2005. A molecular basis for NO selectivity in soluble guanylate cyclase. *Nature Chemical Biology* **1**: 53–59.

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ. 2000. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant Journal* **24**: 667–677.

Clarke GM, Higgins TN. 2000. Laboratory investigation of hemoglobinopathies and thalassemias: review and update. *Clinical Chemistry* **46**: 1284–1290.

Cousson A. 2001. Pharmacological evidence for the implication of both cyclic GMP-dependent and -independent transduction pathways with auxin-induced stomatal opening in *Commelina communis* (L.). *Plant Sciences* **161**: 249–258.

Delledonne M. 2005. NO news is good news for plants. *Current Opinion in Plant Biology* **8**: 390–396.

Delledonne M, Xia Y, Dixon RA, Lamb C. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**: 585–588.

Desikan R, Reynolds A, Hancock JT, Neill SJ. 1998. Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures. *Biochemical Journal* **330**: 115–120.

Donaldson L, Ludidi N, Knight MR, Gehring C, Denby K. 2004. Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. *FEBS Letters* **569**: 317–320.

Durner J, Wendehenne D, Klessig D. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-Ribose. *Proceedings of the National Academy of Sciences, USA* **95**: 10328–10333.

Ederli L, Meier S, Borgogni A, Reale L, Ferranti F, Gehring C, Pasqualini S. 2008. cGMP in ozone and NO-dependent responses. *Plant Signalling & Behavior* **3**: 36–37.

Ederli L, Morettini R, Borgogni A, Westernack C, Miersch O, Reale L, Ferranti F, Tosti N, Pasqualini S. 2006. Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants. *Plant Physiology* **142**: 595–608.

Grennan AK. 2007. Protein *S*-nitrosylation: potential targets and roles in signal transduction. *Plant Physiology* **144**: 1237–1239.

Hoshi T. 1995. Regulation of voltage dependence of the KAT1 channel by intracellular factors. *The Journal of General Physiology* **105**: 309–328.

Hu X, Neill SJ, Tang Z, Cai W. 2005. Nitric oxide mediates gravitropic bending in soybean roots. *Plant Physiology* **137**: 663–670.

Iglesias DJ, Calatayud A, Barreno E, Primo-Millo E, Talon M. 2006. Responses of citrus plants to ozone: leaf biochemistry, antioxidant mechanisms and lipid peroxidation. *Plant Physiology and Biochemistry* **44**: 125–131.

Jabs T, Dietrich RA, Dangl JL. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* **273**: 1853–1856.

Jourd'heuil D. 2002. Increased nitric oxide-dependent nitrosylation of 4,5-diaminofluorescein by oxidant: implications for the measurement of intracellular nitric oxide. *Free Radical Biology & Medicine* **33**: 676–684.

Kangasjarvi J, Talvinen J, Utriainen M, Karjalainen R. 1994. Plant defence systems induced by ozone. *Plant, Cell & Environment* **17**: 783–794.

Kwezi L, Meier S, Mungur L, Ruzvidzo O, Irving H, Gehring C. 2007. The *Arabidopsis thaliana* brassinosteroid receptor (AtBRI1) contains a domain that functions as a guanylyl cyclase *in vitro*. *PLoS ONE* **2**: e449.

Leng Q, Mercier RW, Yao W, Berkowitz GA. 1999. Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. *Plant Physiology* **121**: 753–761.

Liu Y, Ruoho A, Rao V, Hurley J. 1997. Catalytic mechanisms of the adenyl and guanylyl cyclases: modelling and mutational analysis. *Proceedings of the National Academy of Sciences, USA* **94**: 13414–13419.

Ludidi N, Gehring C. 2003. Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **8**: 6490–6494.

Ma W, Berkowitz GA. 2007. The grateful dead: calcium and cell death in plant innate immunity. *Cellular Microbiology* **9**: 2571–2585.

Maathuis FJM. 2006. cGMP modulates gene transcription and cation transport in *Arabidopsis* roots. *Plant Journal* **45**: 700–711.

Meier S, Gehring C. 2006. Emerging roles in plant biotechnology for the second messenger cGMP – guanosine 3′,5′-cyclic monophosphate. *African Journal of Biotechnology* **5**: 1687–1692.

Meier S, Seoighe C, Kwezi L, Irving H, Gehring C. 2007. Plant nucleotide cyclases: an increasingly complex and growing family. *Plant Signalling & Behavior* **2**: 536–539.

Murphy ME, Noack E. 1994. Nitric oxide assay using hemoglobin method. *Methods in Enzymology* **233**: 240–250.

Neill S, Bright J, Desikan R, Hancock J, Harrison J, Wilson I. 2007. Nitric oxide evolution and perception. *Journal of Experimental Botany* **59**: 25–35.

Neill SJ, Desikan R, Hancock JT. 2003. Nitric oxide signalling in plants. *New Phytologist* **159**: 11–35.

Newton RP, Smith CJ. 2004. Cyclic nucleotides. *Phytochemistry* **65**: 2423–2437.

Ogawa D, Nakajima N, Sano T, Tamaoki M, Aono M, Kubo A, Kanna M, Ioki M, Kamada H, Saji H. 2005. Salicylic acid accumulation under O_3 exposure is regulated by ethylene in tobacco plants. *Plant and Cell Physiology* **46**: 1062–1072.

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L. 2002. Nitric oxide is required for root organogenesis. *Plant Physiology* **129**: 954–956.

Pasqualini S, Della Torre G, Ferranti F, Ederli L, Piccioni C, Reale L, Antonielli M. 2002. Salicylic acid modulates ozone-induced hypersensitive cell death in tobacco plants. *Physiologia Plantarum* **115**: 204–212.

Pasqualini S, Piccioni C, Reale L, Ederli L, Della Torre G, Ferranti F. 2003. Ozone-induced cell death in tobacco cultivar BelW3 plants. The role of programmed cell death in lesion formation. *Plant Physiology* **133**: 1122–1134.

Penson SP, Schuurink RC, Fath A, Gubler F, Jacobsen JV, Jones RL. 1996. cGMP is required for gibberellic acid-induced gene expression in barley aleurone. *Plant Cell* **8**: 2325–2333.

Pharmawati M, Billington T, Gehring CA. 1998a. Stomatal guard cell responses to kinetin and natriuretic peptides are cGMP dependent. *Cellular and Molecular Life Sciences* **54**: 272–276.

Pharmawati M, Gehring CA, Irving HR. 1998b. An immunoaffinity purified plant natriuretic peptide analogue modulates cGMP level in the *Zea mays* root stele. *Plant Science* **137**: 107–115.

Prado AM, Porterfield DM, Feijo JA. 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development* **131**: 2707–2714.

Rao MV, Lee HI, Davis KR. 2002. Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. *Plant Journal* **32**: 447–456.

Sabar M, De Paepe R, de Kouchkovsky Y. 2000. Complex I impairment, respiratory compensations, and photosynthetic decrease in nuclear and mitochondrial male sterile mutants of *Nicotiana sylvestris*. *Plant Physiology* **124**: 1239–1249.

Salmi ML, Morris KE, Roux SJ, Porterfield DM. 2007. Nitric oxide and cGMP signalling in calcium-dependent development of cell polarity in *Ceratopteris richardii*. *Plant Physiology* **144**: 94–104.

Shapiro AD. 2005. Nitric oxide signalling in plants. *Vitamins & Hormones* **72**: 339–398.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**: 562–565.

Zeier J, Delledonne M, Mishina T, Severi E, Sonoda M, Lamb C. 2004. Genetic elucidation of nitric oxide signalling in incompatible plant–pathogen interactions. *Plant Physiology* **136**: 2875–2886.

Zhang A, Jiang M, Zhang J, Ding H, Xu S, Hu X, Tan M. 2007. Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defence in maize leaves. *New Phytologist* **175**: 36–50.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Accumulation of cyanide developed from 5 mM SNP solution measured with a cyanide detection kit.

Fig. S2 Cell death evaluated by histochemical staining with trypan blue.

Table S1 Primer sequences of genes tested

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