Effects of modifying alternative respiration on nitric oxide-induced virus resistance and PR1 protein accumulation

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Nitric oxide (NO) is an important defensive signal in plants but its effects on virus infection are not well understood. Administration of NO-releasing compounds immediately before inoculation of tobacco leaves with potato virus X and tobacco mosaic virus decreased the accumulation of virus, indicating that NO can induce resistance rapidly. Resistance induction was inhibited by co-administration with an NO-scavenging compound or when experiments were done in transgenic tobacco plants expressing increased alternative respiratory pathway capacity due to constitutive expression of the plant mitochondrial enzyme, alternative oxidase (AOX). These results indicate that NO, which inhibits electron transport chain activity, is triggering defensive signalling by inducing changes in mitochondrial reactive oxygen species levels that are in turn regulated by AOX. Experiments using nahG-transgenic plants, which cannot accumulate the defensive plant hormone salicylic acid (SA) showed that NO rapidly induces resistance to virus infection independently of SA. However, this initial state of resistance may be transient. Subsequently, by 5 days post-treatment, NO had caused an increase in pathogenesis-related protein 1 (PR1) expression (a proxy for increased SA biosynthesis), which correlated with a longer-term state of resistance to virus infection. The induction by NO of PR1 accumulation was modified in AOX-transgenic plants. This indicates that the influence of NO on defensive gene expression is in part mediated through its effects on mitochondria.

INTRODUCTION

The gaseous hormone nitric oxide (NO) is a key regulator of defence and stress responses in plants and animals (Moreau et al., 2010; Gupta et al., 2011). In plants, NO is an important component in defensive signalling processes that occur in the wake of a hypersensitive response (HR), a relatively rapid response in which potential pathogens are trapped in the vicinity of the infection site (Durner et al., 1998; Delledonne et al., 1998). The HR is typically conditioned by the presence of a dominant resistance (R) gene in the plant and a corresponding avirulence (Avr) gene in the pathogen (Moffett, 2009). The best understood of these gene-for-gene relationships are explained mechanistically by effector-triggered immunity, in which the R gene product allows the plant to perceive damage caused by a pathogenicity factor (effector) encoded by a pathogen Avr gene (Moffett, 2009). This recognition event activates the earliest events in the HR and can lead eventually to the induction of stronger plant-wide enhancements in defensive readiness, such as systemic acquired resistance (SAR) and the accumulation of defence-related proteins such as pathogenesis-related (PR) protein 1 (Carr et al., 2010; Palukaitis et al., 2013).

In plants, NO is known to initiate defensive signalling by at least two mechanisms, including activation of guanylate cyclase to initiate cGMP-mediated signal transduction (Durner et al., 1998) and post-translational protein modification through S-nitrosylation (Feechan et al., 2005), which among other things may aid in regulation of non-expressor of PR protein 1 (NPR1), an important regulator of defence gene transcription (Tada et al., 2008), and in the regulation of an NADPH oxidase that controls HR-associated programmed host cell death (Yun et al., 2011). Meanwhile, it is likely that additional modes of action for NO remain to be discovered in plants (Moreau et al., 2010; Gupta et al., 2011).

In defensive signal transduction NO appears to lie upstream of salicylic acid (SA), another important stress hormone in plants that is an essential signal in the induction of SAR and restriction of pathogens during the HR (Carr et al., 2010; Scheler et al., 2013). In many respects, SA-induced resistance to cellular pathogens (bacteria, fungi and oomycetes) remains
better understood than SA-induced resistance to viruses (Palukaitis et al., 2013). Similarly, the majority of studies of the role of NO in plant defence have focused on host interactions with non-viral pathogens. However, previous reports showed that application of NO-releasing compounds enhances resistance to tobacco mosaic virus (TMV) in tobacco (Song and Goodman, 2001) and that in a TMV-susceptible variety of tomato, endogenous NO levels increase throughout the plant upon infection with this virus (Fu et al., 2010). Similar systemic effects have been seen with increased reactive oxygen species (ROS) production in virus-infected Arabidopsis thaliana plants, indicating the existence of complex ROS and NO signalling events occurring even in susceptible hosts (Love et al., 2005).

A convergence point between signalling mediated by NO and SA is the mitochondrion. Both signal chemicals affect mitochondrial respiratory metabolism by stimulating increased ROS production and enhancing activity and gene expression for the alternative oxidase (AOX) (Ederli et al., 2006; Huang et al., 2002; Norman et al., 2004). AOX is the sole component of the alternative respiratory pathway (AP), which is a plant-specific branch of the mitochondrial electron transport chain that possesses a number of functions including the maintenance of cellular homeostasis and the negative regulation of ROS production (Affourtit et al., 2001). ROS are produced constantly as a by-product of respiratory chain activity but in addition to their toxic effects they can initiate retrograde signalling between the mitochondrion and nucleus. This is likely through effects on glutathione metabolism and redox-sensitive proteins in the mitochondrion (Mailloux et al., 2013; Dutilleul et al., 2003). The plant mitochondrion is also a site of NO production (Gupta et al., 2012). Wulff et al. (2009) showed that, in addition to diminishing ROS levels in plant mitochondria, AOX also decreases NO levels. NO inhibits cytochrome c oxidase, causing an increase in ROS and triggering increased AOX expression and AOX activity (Huang et al., 2002). Unlike cytochrome c oxidase activity, AOX activity is not inhibited by NO (Huang et al., 2002). A plausible interpretation of these findings is that AOX is a regulator of NO-mediated signalling in plant mitochondria. AOX has been shown to affect SA-induced resistance to viruses, likely through its regulatory effects on ROS-triggered retrograde signalling and defensive signalling (Gilliland et al., 2003; Murphy et al., 2004; Singh et al., 2004; Love et al., 2007; Lee et al., 2011; Ma et al., 2011; Zhang et al., 2012). It seemed interesting, therefore, to investigate whether NO-induced resistance to virus infection is influenced by AOX.

RESULTS

Altering AP capacity affects NO-induced resistance to Potato virus X

To investigate the effects of altering AP capacity on NO-induced resistance to PVX, leaves of Aox-transgenic tobacco plants of previously described lines Sn21 (with increased AP capacity) and Sn10 (decreased AP capacity) (Gilliland et al., 2003) were inoculated with PVX immediately after infiltration with SA, the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) or water (Fig. 1a). Treatment with SNAP inhibited PVX accumulation in inoculated leaves of non-transgenic tobacco plants (Fig. 1a). SNAP-induced resistance to PVX was not seen when 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an NO scavenger, was present. This confirms that SNAP-induced resistance to the virus was triggered by the release of NO from SNAP and not from some secondary pharmacological effect of SNAP. In plants of line Sn10, SNAP induced resistance to PVX while in plants of line Sn21 SNAP-induced resistance to PVX was inhibited (Fig. 1a). Similar results were found with PVX when another NO donor, (Z)-1,N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA NONOate), was used to treat non-transgenic, Sn10 and Sn21 plants (Fig. 1b) and when plants were challenged with TMV following treatment with the NO donor S-nitrosoglutathione (GSNO) (Fig. S1, available in the online Supplementary Material). Thus, NO induces resistance to PVX and NO-induced resistance to the virus can be modulated by altering AP capacity.

Altering AP capacity affects PR1 protein accumulation induced by NO but not by SA

To examine the effects of altering AP capacity on NO-induced PR1 protein accumulation, non-transgenic tobacco plants and plants of lines Sn10 and Sn21 were treated with SNAP at various concentrations (2.0, 1.5, 1.0 and 0.5 mM), with 1 mM SA, or with water. In non-transgenic tobacco, PR1 accumulation was detectably increased by application of 1.0 mM SNAP and could be further increased by treatment with 1.5 mM SNAP (Fig. 2a). Application of 2.0 mM SNAP caused no further increase in PR1 accumulation and in no cases did SNAP cause an increase in PR1 accumulation of more than ca 20% of that caused by application of 1 mM SA (Figs 2a and S2a). However, in plants of line Sn10, which has a decreased AP capacity, the accumulation of PR1 in response to 2.0, 1.5 and 1 mM SNAP was increased; with PR1 accumulation in tissues treated with 2.0 or 1.5 mM SNAP reaching approximately 40% of the level of PR1 induced by SA (Figs 2a and S2a). In plants of line Sn21, which has an elevated AP capacity, SNAP-induced PR1 accumulation was drastically decreased at all SNAP concentrations tested (Fig. 2a). In contrast, altering AP capacity did not inhibit or stimulate SA-induced PR1 accumulation either at 1 mM or when SA was applied across a range of lower concentrations (0.5, 0.25, 0.10 and 0.05 mM) (Figs 2a, b and S2b). The resistance-inducing SA functional analogue, 2,6-dichloroisonicotinic acid (INA; Vernooij et al., 1995), which was used as a positive control in some experiments, also appeared to be unaffected in its ability to induce PR1 accumulation in plants that had an altered AP capacity (Fig. 2). The results show that AP capacity affects induction of defence gene expression by NO but not by SA or INA.
The role of SA in NO-induced resistance to PVX

In an earlier study it was found that induction of virus resistance by NO-releasing chemicals was an SA-dependent process (Song & Goodman, 2001). However, in that work there was a delay of 5 days between the application of the chemical inducer and virus inoculation, whereas in the present study we have described experiments in which inoculation was carried out immediately following infiltration with chemical solutions (Figs 1 and 2). In preliminary experiments we noted that the induction of PR1 accumulation by an NO-releasing chemical occurs more slowly than induction by SA (Fig. S3). The accumulation of PR1 is a reliable proxy for the accumulation of SA in plant tissues (Zhou et al., 2014). Thus, we conclude that the induction of PR1 gene expression, which is regulated by SA, cannot occur until sufficient endogenous SA has accumulated in response to the exogenously applied NO. Leaves of non-transgenic and NahG-transgenic tobacco plants were infiltrated with 2 mM SNAP, 1 mM SA, 1 mM INA, or water and challenged by inoculation with PVX on the infiltrated leaves either at 5 days post-treatment (Fig. 3a) or immediately after treatment (Fig. 3b). When inoculation with PVX was carried out at 5 days post-treatment, neither SNAP nor SA induced resistance to the virus in leaves of NahG-transgenic tobacco, whereas resistance to PVX was induced by INA, which is not broken down by the SA hydroxylase encoded by the NahG transgene (Fig. 3a). In contrast, when PVX was inoculated into leaves immediately after chemical treatment, PVX accumulation was inhibited in SNAP-treated NahG-transgenic tobacco leaves, indicating that NO can rapidly induce resistance to the virus in an SA-independent fashion (Fig. 3b).

DISCUSSION

The results show that NO can rapidly induce resistance to virus infection independently of SA. This initial state of resistance appears to be transient, however, and exposure to exogenously applied NO leads eventually to an increase in PR1 accumulation (diagnostic for increased SA biosynthesis), indicating the establishment of a longer-term state of resistance to virus infection (Fig. 4). We believe it was this longer-term, SA-dependent resistance that was observed previously in the study by Song & Goodman (2001). The timing of this second phase of resistance is commensurate with the pattern of induction of PR1 gene expression by NO-releasing chemicals, which was slower than that induced by application of exogenous SA and is likely to have depended on the upregulation of synthesis of SA by the plant.

The results with transgenic plants possessing altered AP capacity indicate that the rapid induction of resistance to virus infection by NO-releasing compounds likely starts with NO-mediated inhibition of cytochrome c oxidase leading to a transient increase in mitochondrial ROS. As previously theorized for induction of resistance to viruses by other cytochrome pathway inhibitors such as cyanide and antimycin A, we suggest that this is the first stage in a process of retrograde signalling from the mitochondrion to the nucleus, where changes in gene expression occur (Chivasa & Carr, 1998; Gilliland et al., 2003; Singh et al., 2004; Lee et al., 2011). This ROS increase will be countered by activation of anti-oxidant systems including the activation of AOX and the consequent engagement of the AP; in other words, AOX and the AP act as negative regulators of this form of
defensive signalling (Gilliland et al., 2003; Singh et al., 2004; Lee et al., 2011). The presence of NO in the plant will also lead to the induction of SA biosynthesis and induction of the longer-term state of resistance, which was previously observed by Song & Goodman (2001) (Fig. 4). NO has been identified as an inducer of RNA-dependent RNA polymerase 1 (RDR1), which has antiviral effects (Liao et al., 2013). However, RDR1 gene induction is not affected by AOX-regulated signalling (Gilliland et al., 2003) and it is likely that NO-induced RDR1 induction is triggered

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**Fig. 2.** Effects of altering alternative respiratory capacity on NO-induced PR1 accumulation in tobacco plants. Immunoblot analysis (using anti-PR1) of proteins extracted from leaves of non-transgenic plants or Aox-transgenic plants of lines Sn10 or Sn21 (with decreased or increased AP capacity, respectively, indicated by down- or upwards pointing arrows). Leaves had been infiltrated 5 days previously with the indicated concentrations of SNAP (a) or SA (b). Control leaves were infiltrated with water or with 1 mM SA (a) or 2,6-dichloroisonicotinic acid (INA) (b). Anti-PR1 binding was detected using goat anti-rabbit IgG conjugated to IRDye 800CW and secondary antibody binding was imaged using a Li-Cor Odyssey infrared scanner. Ponceau S staining of the filter was used to check equal protein loading: the band for the large subunit (LSU) of ribulose 1,5-bisphosphate carboxylase is shown in the lower panels.

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**Fig. 3.** NO can induce rapidly manifested, SA-independent and slower, SA-dependent resistance to PVX. Leaves of non-transgenic (NT) or NahG-transgenic tobacco plants were infiltrated with water, 2 mM SNAP, 1 mM INA or 1 mM SA. Leaves were inoculated with PVX either at 5 days post-infiltration (a) or immediately following chemical infiltration (b). In both cases, protein was extracted for immunoblot analysis of PVX coat protein (PVX CP) accumulation 5 days following virus inoculation. Binding of anti-PVX CP was detected using goat anti-rabbit IgG conjugated to IRDye 800CW and its binding was imaged using a Li-Cor Odyssey infrared scanner. Equal loading was checked by Ponceau S staining of the filter and the band for the large subunit (LSU) of ribulose 1,5-bisphosphate carboxylase is shown in the lower panels.
indirectly by the stimulation of SA biosynthesis or effects on several other phytohormones known to induce RDR1 expression (Pandey & Baldwin, 2007; Hunter et al., 2013; Xu et al., 2013). Thus, it is unlikely that RDR1 is involved in the rapid phase of resistance induction but it could well be involved in the slower, SA-dependent phase.

During this work it was observed that NO-induced accumulation of PR1 (an indicator for resistance induction but not directly associated with virus resistance; Carr et al., 2010) was affected in transgenic plants with altered AP capacity. Indeed it appears from these results that AOX and other genes result in increased SA biosynthesis, leading to a more sustained resistance to viruses (labelled ‘slow’), as well as to the triggering of increased expression of defence genes such as PR1. Lines indicating feed-back from SA to the nucleus and mitochondrion (where it influences ROS formation and gene expression, respectively) have been omitted from this model for clarity.

**Fig. 4.** The effects of NO on induced virus resistance and defence gene induction. Our data indicate that administration of NO-releasing compounds stimulates ROS accumulation in mitochondria and that this is countered by the activity of AOX, the sole component of the plant AP. We suggest that changes in mitochondrial ROS initiate retrograde signalling to the nucleus, where changes in gene expression occur, of which some lead rapidly (and independently of SA) to the induction of resistance to viruses (labelled ‘rapid’, and others result in increased SA biosynthesis, leading to a more sustained resistance to viruses (labelled ‘slow’), as well as to the triggering of increased expression of defence genes such as PR1. Lines indicating feed-back from SA to the nucleus and mitochondrion (where it influences ROS formation and gene expression, respectively) have been omitted from this model for clarity.

priming step or affecting the redox state of NPR1), in addition to its direct and indirect effects on other targets in defence exerted through nitrosylation (reviewed by Mur et al., 2013).

**METHODS**

**Plant lines and growth conditions.** Non-transgenic tobacco (Nicotiana tabacum L.) cv. Xanthi and Xanthi-nc (NN genotype), NahG-transgenic tobacco (line 10, Xanthi-nc background) (Gaffney et al., 1993) and AOX-transgenic tobacco lines Sn10 and Sn21 (Xanthi background) (Gilliland et al., 2003) were raised in a growth room (Conviron) maintained at 22 °C and 60 % relative humidity under a 16 h photoperiod (200 μE m−2 s−1 photosynthetically active radiation).

**Chemical treatments and virus inoculation.** Stock solutions of SA (Sigma-Aldrich) and INA (Maybridge Chemical) were prepared in water, filter sterilized and stored at −20 °C. Before application to plant tissue, SA and INA stock solutions (10 mM) were diluted to 1 mM in sterile filtered water. Various concentrations of NO reagents were prepared freshly in sterile filtered water. These included the NO donors DEA-NONOate (1 mM), SNAP (2, 1.5, 1 or 0.5 mM), GSNO (0.5 or 0.25 mM) and the NO scavenger cPTIO (0.5 mM). NO-releasing and -scavenging reagents were obtained from Enzo Life Sciences, except for GSNO, which was obtained from Calbiochem (now Merck-Millipore). For treatment of plant tissue, water (control) or reagent solutions were infiltrated through the lower epidermis of expanded leaves of 4- to 5-week-old transgenic and non-transgenic tobacco plants into the intercellular space using a syringe without a needle. Leaves were inoculated with aqueous suspensions (10 μg ml−1) of PVX (UK3 strain; Köhm et al., 1993) or TMV (U1 strain) uniformly over their adaxial surface using a frosted glass slide and carborundum 5 days after or immediately after chemical infiltration. Total soluble protein was extracted as previously described (Lee et al., 2011). Experiments were done at least three times to ensure reproducibility.

**Detection of PVX coat protein and PR1 protein accumulation.** Immunoblot analysis of TMV coat protein accumulation and of PR1 protein accumulation (in initial experiments) was carried out using previously described antisera, and primary antibody binding was detected using anti-rabbit IgG conjugated to horseradish peroxidase and a chemiluminescent substrate (Western Lightning; Perkin-Elmer) (Lee et al., 2011; Zhou et al., 2014). In other analyses of PR1 accumulation and for detection of PVX coat protein accumulation, primary antibody binding was detected with goat anti-rabbit IgG (H + L)-conjugated IRDye 800CW (Li-COR Biosciences) at 1:15 000 dilution, imaged using the Odyssey quantitative infrared imaging system (ODYSSEY CLx; Li-COR Biosciences) and quantified using the Odyssey Image Studio Software package (Li-COR). Anti-PVX coat protein and anti-TMV coat protein sera were used at dilutions of 1:15 000 and anti-PR1 serum at 1:10 000 in PBS.

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