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# Coordination of a mitochondrial superoxide burst during the hypersensitive response to bacterial pathogen in *Nicotiana tabacum*

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## ABSTRACT

**We characterized responses of *Nicotiana tabacum* to pathovars of the bacterial pathogen *Pseudomonas syringae*. These included a compatible response associated with necrotic cell death (pv. *tabaci*), an incompatible response that included hypersensitive response (HR) cell death (pv. *maculicola*) and an incompatible response that induced defences but lacked the HR (pv. *phaseolicola*). Signalling molecules (salicylic acid, nitric oxide, $H_2O_2$) known to induce the stress responsive tobacco *Aox1a* gene [that encodes the mitochondrial electron transport chain (ETC) component alternative oxidase (AOX)] accumulated preferentially during the HR, but this did not elevate *Aox1a* transcript or AOX protein, while the transcript and protein were strongly elevated during the defence response to pv. *phaseolicola*. In addition, matrix manganese superoxide dismutase (MnSOD) activity declined during the HR, unlike its response to the other pathovars, and unlike the response of other superoxide dismutase (SOD) enzymes. Finally, the HR (but not the response to pv. *phaseolicola* or pv. *tabaci*) was accompanied by an early and persistent mitochondrial superoxide ($O_2^-$) burst prior to cell death. We propose that a coordinated response of the major ETC mechanism to avoid $O_2^-$ generation (AOX) and the sole enzymatic means to scavenge mitochondrial $O_2^-$ (MnSOD) is important in the determination of cell fate during responses to pathogen.**

*Key-words*: alternative oxidase; manganese superoxide dismutase; mitochondrion; nitric oxide; programmed cell death; *Pseudomonas syringae*; salicylic acid; superoxide; tobacco.

*Abbreviations*: ACC oxidase, 1-aminocyclopropane-1-carboxylic acid oxidase; AOX, alternative oxidase; CuZnSOD, copper zinc superoxide dismutase; cyt, cytochrome; ETC, electron transport chain; FeSOD, iron superoxide dismutase; HR, hypersensitive response; MnSOD, manganese superoxide dismutase; NO, nitric oxide; $O_2^-$, superoxide; $ONOO^-$, peroxynitrite; PCD, programmed cell death; *PR-1*, pathogenesis-related-1; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SA, salicylic acid; SAG, salicylic acid $\beta$-glucoside; SOD, superoxide dismutase.

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## INTRODUCTION

Infection of a plant by a pathogen has two possible outcomes – disease (compatible interaction) or resistance (incompatible interaction). During one type of incompatible interaction, recognition of the pathogen by the plant triggers the HR, a localized PCD thought to delay pathogen spread (Coll, Eppie & Dangl 2011). Even though this host-controlled process may not be essential for resistance, it is required for the rapid and strong activation of other defence responses (Heath 2000). Another type of incompatible interaction occurs if the pathogen fails to overcome even basal defence barriers and fails to proliferate. While such an interaction does not induce the HR, it does nonetheless induce other defence responses (Mysore & Ryu 2004). In a typical compatible interaction, the pathogen is able to suppress or overcome basal and induced plant defences, thus successfully colonizing the host and causing disease (Nomura, Melotto & He 2005).

Recent research has established that the mitochondrion is likely an important player in plant PCD, including the HR, although the details of its role are not well understood and expected to differ from that seen in animals (Lam, Kato & Lawton 2001; Amirsadeghi, Robson & Vanlerberghe 2007; Noctor, De Paepe & Foyer 2007; van Doorn *et al.* 2011). An important emerging theme, however, is that the mitochondrion may be an important early source of intracellular ROS, a so-called mitochondrial oxidative burst. This is accompanied by changes in mitochondrial membrane potential, the mitochondrial permeability transition, cyt *c* release (at least in some cases) and changes in mitochondrial morphology (Arpagaus, Rawyler & Braendle 2002; Tiwari, Belenghi & Levine 2002; Yao *et al.* 2002, 2004; Weir, Pham & Hedley 2003; Takahashi *et al.* 2004; Ashtamker *et al.* 2007; Garmier *et al.* 2007). In some cases, blockage of one or more of these events, such as inhibition of the permeability transition by cyclosporine A, is shown to block or delay PCD. Nonetheless, it remains obscure what overall role and influence these mitochondrial events have in PCD.

Besides cyt oxidase, plants have an additional terminal oxidase in the mitochondrial ETC called AOX that catalyses the oxidation of ubiquinol and reduction of $O_2$ to $H_2O$, and which has been linked to the control of ROS generation by the ETC (Finnegan, Soole & Umbach 2004; Vanlerberghe, Cvetkovska & Wang 2009). AOX is non-proton pumping and since it bypasses proton-pumping complexes III and IV, electron flow to AOX dramatically reduces the energy yield of respiration. AOX is encoded by a small nuclear gene family and is an interfacial membrane protein on the matrix side of the inner membrane. Many AOX members contain a conserved regulatory Cys residue that confers tight biochemical control, linking AOX activity to prevailing metabolic conditions. Because of its non-energy conserving nature, AOX reduces the otherwise tight coupling between carbon metabolism, electron transport and ATP turnover. This flexibility in metabolism is likely important in numerous contexts, but particularly under stress conditions (Finnegan *et al*. 2004; Vanlerberghe *et al*. 2009). A central hypothesis is that AOX acts to dampen $O_2^-$ generation by the ETC, since it bypasses Complex III, an important source of $O_2^-$, and since its energy-dissipating nature will act to moderate membrane potential. This will lower the overall reduction state of ETC components, lessening the electron leak that produces $O_2^-$ (Møller 2001). Supporting this, chemical inhibition of AOX or assay conditions that do not favour the activation of AOX result in higher rates of ROS generation by isolated mitochondria (Purvis 1997; Braidot *et al*. 1999). Furthermore, in transgenic tobacco suspension cells, silencing of AOX was accompanied by increased ROS that appeared to emanate specifically from the mitochondria (Maxwell, Wang & McIntosh 1999).

SA, NO and ROS such as $H_2O_2$ and $O_2^-$ are important signalling molecules to initiate and coordinate defence responses, including the HR, during incompatible interactions (Leitner *et al*. 2009; Vlot, Dempsey & Klessig 2009; Spoel & Loake 2011). Interestingly, strong links exist between these signalling molecules and mitochondria. A low level of exogenously applied SA can disrupt the ETC, although the mechanism(s) involved is poorly understood (Norman *et al*. 2004; de Souza *et al*. 2011). Perhaps due to this disruption, SA dramatically induces the level of AOX transcript and protein in many species (Rhoads & McIntosh 1992; Van Der Straeten *et al*. 1995; Ho *et al*. 2008). NO is a well-known competitive inhibitor of cyt oxidase, but AOX is not subject to this inhibition (Millar & Day 1996), and exogenous applied NO has been demonstrated to be an effective inducer of AOX gene expression (Huang, von-Rad & Durner 2002; Ederli *et al*. 2006). What is not clear is whether the ability of NO to induce AOX results primarily from it acting as a respiratory inhibitor or whether NO can also induce AOX through its action as a signalling molecule, independent of inhibition of cyt oxidase. $O_2^-$ is a well-established by-product of ETC activity and, once produced, can be scavenged and converted to $H_2O_2$ by a mitochondrion-specific and matrix-localized MnSOD (Møller 2001). Finally, exogenous $H_2O_2$ can induce AOX gene expression (Vanlerberghe & McIntosh 1996), and $H_2O_2$-responsive elements are present in the stress-responsive *Aox1a* gene of *Arabidopsis* (Ho *et al*. 2008).

Disparate studies have investigated the role of mitochondrial ROS in PCD, the role of AOX in avoiding ROS generation and links between mitochondrial function and biotic stress signalling molecules. Based on these studies, we proposed that AOX might be an important determinant of cell fate during responses to pathogens. To robustly test this hypothesis, we characterized the response of *Nicotiana tabacum* to pathovars of *Pseudomonas syringae*. Based on our results, we propose that a coordinated response of the major ETC mechanism to avoid $O_2^-$ generation (AOX) and the sole enzymatic means to scavenge mitochondrial $O_2^-$ (MnSOD) is important in the determination of cell fate during responses to pathogen.

## MATERIALS AND METHODS

### Plant material and growth conditions

*N. tabacum* L. cv. Petit Havana SR1 was grown in controlled-environment chambers (PGR-15, Conviron, Winnipeg, Canada) with a 16 h photoperiod, temperature of 28 °C/22 °C (light/dark), relative humidity of 60%, photosynthetic photon flux density of 130 $\mu$mol $m^{-2}$ $s^{-1}$, and in a general-purpose growing medium (Pro-mix BX, Premier Horticulture, Canada). Plants were irrigated with water daily and fertilized with 10× diluted Hoagland's solution three times per week. *Arabidopsis thaliana* ecotype Columbia was grown similarly but with a 9 h photoperiod, constant temperature (22 °C), relative humidity of 65% and photosynthetic photon flux density of 200 $\mu$mol $m^{-2}$ $s^{-1}$. Plants were grown in the same medium as above and irrigation alternated between water and general-purpose fertilizer (Plant Products, Brampton, Canada). Plants were used 5–6 weeks after initiating germination in vermiculite (tobacco) or soil (*Arabidopsis*). Tobacco suspension cells were derived from leaf tissue and grown heterotrophically as previously described (Robson & Vanlerberghe 2002). Cultures were maintained in the dark on a rotary shaker (140 rpm, 28 °C) and were subcultured every 7 days by dilution in fresh growth medium. Cells (200 mL cultures) were used at 2 days after subculture.

### Bacteria and plant inoculations

*P. syringae* pv. *tabaci*, pv. *maculicola* ES4326, pv. *phaseolicola* NPS3121 and pv. *tomato* strains DC3000, DC3000 (*avrRpt2*) and DC3000 (*avrRps4*) were each acquired from Dr K. Yoshioka, University of Toronto. All bacteria were cultured in King's B medium (King, Ward & Raney 1954), either without antibiotic (pv. *maculicola*), with 50 $\mu$g $mL^{-1}$ rifamycin (pv. *phaseolicola*), or with 50 $\mu$g $mL^{-1}$ each of kanamycin and rifamycin (pv. *tabaci* and pv. tomato). Bacteria were grown overnight with rotation (28 °C, ~20 rpm), washed once and resuspended in distilled water (for tobacco) or 10 mM $MgCl_2$ (for *Arabidopsis*). Culture density was determined by absorbance at 600 nm using a

spectrophotometer (HP8452, Agilent Technologies, Mississauga, Canada). Unless stated otherwise, density was adjusted to $1 \times 10^7$ cfu mL$^{-1}$ for plant inoculations, care being taken to inoculate comparable leaves between plants. Tobacco were inoculated at 5 h into the light period by completely infiltrating with liquid the abaxial side of the second to lowest true leaf, using a syringe with needle. Mock-inoculated plants were infiltrated with distilled water. *Arabidopsis* were inoculated at 1 h into the light period by completely infiltrating the abaxial side of rosette leaves using a syringe without needle. Mock inoculations were carried out with 10 mM $MgCl_2$.

## Bacterial growth *in planta* and leaf cell death

Bacterial growth *in planta* was determined by harvesting three leaf discs (1 cm diameter), homogenizing in 10 mM $MgCl_2$, and plating as a dilution series on King's B medium agar plates supplemented with the appropriate antibiotics. Following incubation (2 d, 28 °C), the number of bacterial colonies was determined. The symptoms of the infection were assessed and compared visually, while leaf cell death was also measured using an ion leakage assay. Ten leaf discs (1 cm diameter) were floated abaxial side up on 10 mL distilled water for 4 h in the dark at room temperature (RT). After incubation, conductivity of the bathing solution was measured with a conductivity meter (CON510, Oakton Instruments, Vernon Hills, IL, USA).

## SA determination

Measurement of SA levels was done according to the method of Mosher *et al*. (2010), with some modifications. Leaf tissue (0.1 g FW) was flash frozen in liquid $N_2$ and extracted twice with 0.8 mL extraction buffer {acetone: 50 mM citric acid [70:30] and 5 $\mu$L of internal standard [($^2H_6$)SA; CDN isotopes, Point-Claire, Quebec, Canada]} in 1.5 mL screw-cap tubes containing 1.4 mm ceramic beads (Qbiogene, Carlsbad, CA, USA) and using a FastPrep FP120 homogenizer (Qbiogene). After centrifugation (14 000 *g*, 3 min, RT), the supernatant was removed to a fresh tube, the acetone was dried and the remaining supernatant was extracted with 0.75 mL diethyl ether and centrifuged (14 000 *g*, 1 min, RT), after which the upper organic phase containing free SA was collected. SAG was extracted from the remaining aqueous phase by acidification with 5 $\mu$L 1 M HCl and hydrolysis at 80 °C for 1 h, followed by diethyl ether extraction, as described above. All samples were passed through a Supelclean LC-NH2 SPE column (Supelco, Bellefonte, PA, USA) equilibrated with ether. The column was washed with 1.2 mL chloroform:*n*-propanol (2:1), and samples were eluted with 1.5 mL ether:formic acid (98:2). After drying under a stream of nitrogen, the pellet was resuspended with 0.1 mL dichloromethane:methanol (80:20) and derived with 2 $\mu$L trimethylsilyl-diazomethane (Sigma-Aldrich, Oakville, Canada) for 20 min at RT. The reaction was terminated by adding 2.1 $\mu$L acetic acid in hexane (1:7.5) and incubating for 30 min at RT. The resulting methyl esters were analysed on a gas chromatograph/mass spectrometer (6890N GC connected to a 5975 mass selective detector; Agilent Technologies, Palo Alto, CA, USA) using a DB-5 capillary column (Agilent Technologies) with flow rate = 1.2 mL min$^{-1}$ and helium carrier gas. The methyl esters were measured using selected-ion monitoring with m/z 153 (SA) and m/z 157 ([$^2H_6$]SA).

## $H_2O_2$ determination

$H_2O_2$ was extracted and measured as previously described (Jiang, Woollard & Wolff 1990) with some modifications. Leaf tissue (0.2 g FW) was flash frozen and ground under liquid $N_2$ in 1 mL of 0.2 M $HClO_4$. The homogenate was incubated (5 min, 4 °C) and centrifuged (10 000 *g*, 10 min, 4 °C), and the supernatant was then removed and neutralized to pH 7–8 with 0.2 M $NH_4OH$, pH 9.5. After centrifugation (3000 *g*, 2 min, 4 °C), the supernatant was passed through an AG 1-X8 resin column (Bio-Rad Laboratories, Mississauga, Canada). Following elution from the column with ice-cold distilled water, 0.2 mL of the eluate was combined with 0.5 mL of an assay reagent (0.5 mM $(NH_4)_2Fe(SO_4)_2$, 50 mM $H_2SO_4$, 0.2 mM xylenol orange, 200 mM sorbitol). Following incubation (45 min, RT), the absorbance at 560 nm was measured using a spectrophotometer. To evaluate the % recovery of $H_2O_2$, 5 $\mu$mol of $H_2O_2$ was added directly to the tissue prior to extraction. Recovery ranged from 90 to 95%.

## NO determination

NO was assayed using the method described by Murphy & Noack (1994), with some modifications. Leaf tissue (0.15 g FW) was flash frozen and ground under liquid $N_2$ in 100 mM potassium phosphate buffer (pH 7.0) containing 0.6% (w/v) insoluble PVP. Following addition of powdered activated carbon, the sample was centrifuged (11 000 *g*, 10 min, 4 °C), and the clarified supernatant was pre-treated for 5 min at RT with 100 U catalase and 50 U SOD (Sigma-Aldrich) to remove ROS. Oxyhaemoglobin was prepared fresh on the day of use by converting 20 mg metHb (Sigma-Aldrich) to Hb using an excess of sodium dithionite (2 mM) in 50 mM Tris pH 7.0. The solution was then oxidized with a stream of $O_2$ and desalted by passing it through Sephadex G-25 column (GE Healthcare, Mississauga, Canada). Following elution with 50 mM Tris pH 7.0, the concentration of $HbO_2$ was determined by absorbance at 415 nm (extinction coefficient 131 mM$^{-1}$ cm$^{-1}$). $HbO_2$ (10 $\mu$M) was then added directly to the pre-treated samples (see above) and incubated (5 min, RT). The conversion of $HbO_2$ to metHb was measured by absorbance at 401 and 421 nm. The concentration of NO was calculated as ($A_{401}HbO_2 - A_{421}$ metHb) using extinction coefficient of 77 mM$^{-1}$ cm$^{-1}$.

## Transcript and protein determination

RNA was extracted from leaves and suspension cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA)

according to the manufacturer's instructions. Partial cDNAs (to be used as hydridization probes in Northern blot analyses) were amplified from tobacco or *Arabidopsis* leaf RNA using an RT-PCR kit (Access RT-PCR, Promega, Madison, WI, USA) and cloned into pGEM-T Easy plasmid (Promega). Care was taken to generate cDNA probes that minimized complementarity with other known gene family members. The cDNAs were excised from the plasmid and gel purified for use as hybridization probes. The primer sequences used for RT-PCR can be found in Supporting Information Table S1. In the case of tobacco *Aox1a*, a cloned full-length cDNA was used as probe (Vanlerberghe & McIntosh 1996). Northern blot analyses were performed as previously described (Sieger *et al*. 2005). After X-ray film development, blots were quantified by densitometry using ImageJ (National Institutes of Health, USA). Isolation of mitochondria from leaves (starting with 35 g of leaf tissue from 10 plants) and immunoblot analysis of mitochondrial proteins (AOX and coxII) were done as previously described (Vanlerberghe *et al*. 1995; Robson & Vanlerberghe 2002).

### Tobacco suspension cell experiments

For treatment of suspension cells, a small volume of SA (from a fresh and filter-sterilized 1 M stock solution) was added directly to 200 mL cultures. Culture viability was determined by microscopic observation of a small aliquot of cells treated with 0.025% Evan's Blue, which is excluded from live cells. At least 400 cells were scored to establish the % viability of a culture. For isolation of genomic DNA, tissue (0.2 g FW) was harvested by vacuum filtration, transferred to 1.5 mL screw-cap tubes and flash frozen in liquid $N_2$. The tissue was homogenized in 0.7 mL extraction buffer [100 mM Tris–HCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl, 2% (v/v) sodium dodecyl sulphate, 2% (v/v) $\beta$-mercaptoethanol, pH 8.0 containing 1% (w/v) PVP-10 added after autoclaving]. DNA isolation and analysis was then done as previously described (Lin *et al*. 2001; Robson & Vanlerberghe 2002), and also utilizing of general molecular biology protocols (Sambrook & Russell 2001).

### SOD activities

All steps were performed at 4 °C. Leaf tissue (0.2 g FW) was flash frozen in liquid $N_2$ and homogenized in 0.2 mL protein extraction buffer (50 mM $KH_2PO_4$, 0.2% Triton X-100, 0.05% $\beta$-mercaptoethanol, 1% [w/v] PVP-25, pH 7.8). Samples were incubated (10 min, 4 °C) and centrifuged (13 000 *g*, 10 min, 4 °C), and then aliquots of the supernatant were stored at –80 °C until use. After determination of protein concentration, SOD activity gel staining was performed as previously described (Beauchamp & Fridovich 1971), with some modifications. Non-denaturing gels were pre-equilibrated in a solution containing 0.05 M $KH_2PO_4$, 1 mM EDTA, pH 7.8, along with the appropriate inhibitors when required (2 mM KCN to inhibit copper zinc SOD [CuZnSOD], 5 mM $H_2O_2$ to inhibit CuZnSOD and iron SOD [FeSOD]). After 30 min, the pre-equilibration solution was replaced with a fresh solution containing 0.24 mM nitroblue tetrazolium, 33.2 $\mu$m riboflavin and 0.2% tetramethylethylenediamine. After incubation (30 min, dark, shaking), the gel was illuminated with fluorescent light (400 $\mu$mol $m^{-2}$ $s^{-1}$) for 5 min, or until the gel became uniformly blue except in positions with SOD activity. Individual SOD activities were then analysed using ImageJ.

### Imaging of mitochondrial $O_2^-$

Imaging of $O_2^-$ was performed using the MitoSOX Red mitochondrial $O_2^-$ indicator (M36008; Invitrogen) that is selectively accumulated by mitochondria, where it is oxidized by $O_2^-$ and becomes fluorescent upon binding to nucleic acid (Robinson, Janes & Beckman 2008). Intact tobacco leaves were inoculated with bacteria as usual. At different times, leaves were removed and the lower epidermis was peeled off. The remaining leaf segments were then floated (30 min, RT, dark) on 3 $\mu$M MitoSOX Red in water. In some cases, leaf was instead loaded (20 min, RT, dark) with 0.35 $\mu$m MitoTracker Red (M7512; Invitrogen), which also selectively accumulates in mitochondria. Samples were then mounted on microscope slides, and the mesophyll cell layer was immediately examined with a LSM510 META laser-scanning confocal microscope (Carl Zeiss, Jena, Germany), with excitation at 488 nm (for MitoSOX Red) or 568 nm (for MitoTracker Red) and detection through a 585–615 nm band-pass filter. Z-series (typically 8–16 $\mu$m in depth with 2 $\mu$m step size) were combined in a maximum intensity projection image using LSM510 imaging software and processed with Adobe Photoshop CS2.

## RESULTS

### Compatible and incompatible responses of tobacco leaf to *P. syringae* infection

To study the role of the mitochondrion in responses to biotic stress, tobacco leaf was inoculated with three different *P. syringae* pathovars. An initial characterization established that the pathovars resulted in three different interactions. In the case of pv. *tabaci*, this naturally occurring virulent pathogen of tobacco proliferated rapidly over the first two days (Fig. 1a). Measures of leaf conductivity indicated no loss of membrane integrity at 1 d post-inoculation but significant loss of membrane integrity (a measure of cell death) by 2 d (Fig. 1b). Visual observations indicated the appearance of water-soaked tissue and cell death lesions by 2 d, both classic symptoms of the wildfire disease caused by virulent *P. syringae* (Fig. 2). The pv. *maculicola* also proliferated rapidly over the first day (Fig. 1a) but, unlike pv. *tabaci*, this was accompanied by significant cell death as well by 1 d (Fig. 1b) and little further proliferation of the bacteria after 1 d. In this case, the leaf displayed dry lesions (the typical appearance of HR lesions) beginning at 1 d and the tissue was not water-soaked (Fig. 2). In the case of pv. *phaseolicola*, it did not proliferate after inoculation and

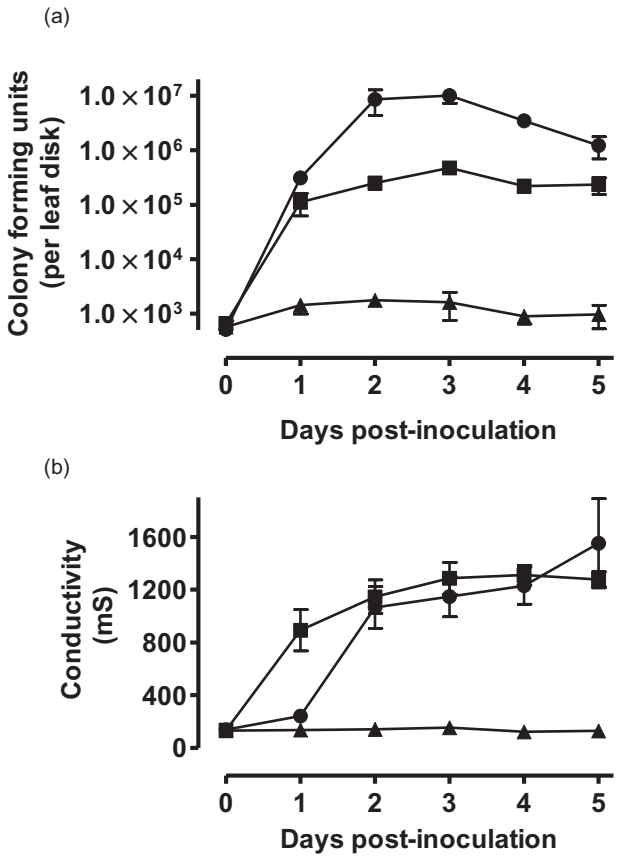


**Figure 1.** Bacterial proliferation in tobacco leaf (a) and conductivity (ion leakage) of tobacco leaf (b) at different times post-inoculation with pathovars of *Pseudomonas syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). For bacterial proliferation experiments, bacterial density for inoculation was adjusted to $1 \times 10^5$ cfu $mL^{-1}$. Data are the mean ± SE of three independent experiments, each of which showed similar results. In some cases, error bars are smaller than the data symbol.

there was no loss of membrane integrity or visual symptoms of cell death or disease (Figs 1 and 2). Summarizing, the results indicate that the interaction of tobacco with pv. *tabaci* is a compatible, disease-causing interaction. The pv. *maculicola* induces a resistance response (incompatible interaction) that includes an HR-like cell death, which we will refer to hereafter as HR. The pv. *phaseolicola* is an incompatible interaction but without cell death.

## Dynamic responses of stress signalling molecules to *P. syringae* infection

To further characterize the plant responses to *P. syringae*, the levels of well-known biotic stress signalling molecules (SA, $H_2O_2$, NO) were quantitatively measured. In the case of infection with HR-inducing pv. *maculicola*, levels of leaf SA increased rapidly between 3 and 6 h post-inoculation (Fig. 3a). The compatible interaction (pv. *tabaci*) had no effect on SA level at 6 h, although some accumulation of SA was seen by 24 h. In the case of infection with the incompatible (but not HR-inducing) pv. *phaseolicola*, some accumulation of SA occurred by 6 h, but to only 36% of the level seen with pv. *maculicola*. No changes were seen in the level of SAG at 6 h post-inoculation with any of the pathovars, but the level of this biologically inactive form did accumulate to high levels by 24 h in response to both of the incompatible interactions (Fig. 3b). Interestingly, the SA accumulation seen with pv. *maculicola* was clearly biphasic being very high at 6 h, back to lower levels at 12 h and higher again by 24 h.

Just as the two incompatible pathovars had different impacts on leaf SA, they also had distinct impacts on leaf $H_2O_2$ and NO levels. Changes in $H_2O_2$ level after inoculation with the HR-inducing pv. *maculicola* were reminiscent of a classic two-phase oxidative burst (with a first and transient peak at 2 h post-inoculation and a second and sustained peak at 8 h), but the variability in this response only allows to conclude that $H_2O_2$ remained high (Fig. 4a). This was accompanied by a rapid 10-fold accumulation of NO over the first 6 h and further accumulation through 24 h (Fig. 4b). In the case of pv. *phaseolicola*, there was instead a general decline in $H_2O_2$ level after inoculation. Similarly, there was no rapid accumulation of NO over the first 6 h or high accumulation by 24 h, but rather just a steady and modest accumulation over the full 24 h period.


**Figure 2.** Typical visual symptoms of tobacco leaf at 2 d post-inoculation with pathovars of *Pseudomonas syringae*. See text for details.

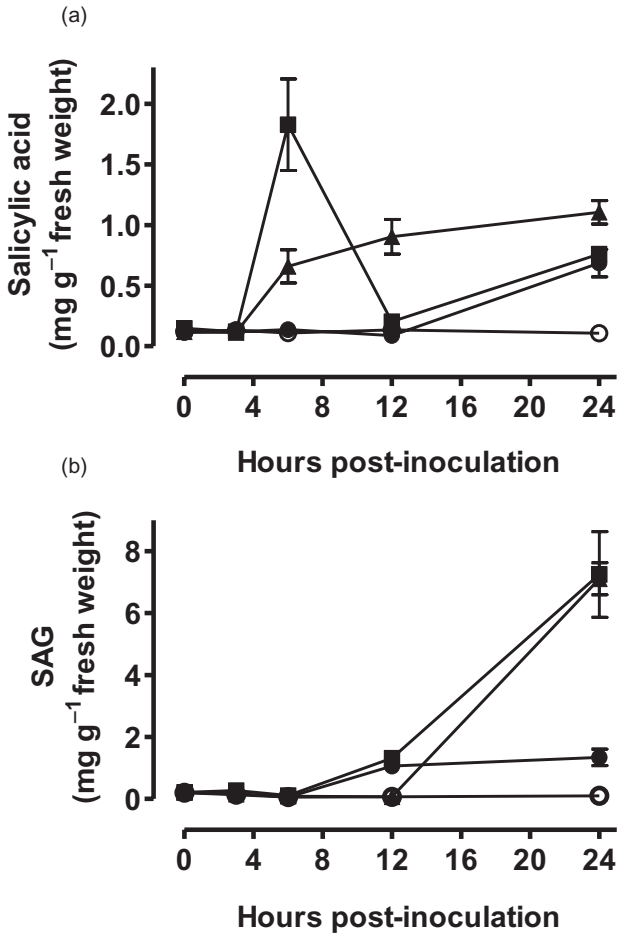


**Figure 3.** Levels of salicylic acid (a) and salicylic acid β-glucoside (SAG) (b) in tobacco leaf at different times post-mock inoculation with $H_2O$ (open circles) or post-inoculation with pathovars of *Pseudomonas syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). Data are the mean ± SE from six separate plants, across two independent experiments. In some cases, error bars are smaller than the data symbol.

## Leaf *Aox1a* transcript levels are dynamic but can be studied in response to bacterial inoculations

We found that the transcript level of tobacco *Aox1a* was extremely dynamic over a diurnal cycle, with transcript levels clearly rising during the early part of the light period and then falling during the later part of the light period and through the dark period (Supporting Information Fig. S1A,B). In contrast, transcript encoding phenylalanine ammonia lyase (PAL) peaked during the dark period (Supporting Information Fig. S1C,D). Therefore, to robustly examine *Aox1a* gene expression in response to inoculation with bacteria, it was critical to always perform inoculations at a defined time (5 h into light period) and to normalize all transcript data against control samples (water-infiltrated leaves) taken in parallel with all treatment samples. It was also important to determine that water infiltration alone was not having a significant effect. To confirm this, *Aox1a* and *PAL* transcript levels were compared in leaves either infiltrated with water or left untreated. Based on these experiments, it was determined that the water infiltration itself was having little, if any, impact (Supporting Information Fig. S1).

## Distinct changes in *Aox1a* transcript level and AOX protein level occur in response to different *P. syringae* pathovars

The level of tobacco *Aox1a* transcript was compared up to 48 h after inoculation with different pathovars and also compared with changes in AOX protein level in isolated tobacco leaf mitochondria (Fig. 5, Supporting Information Fig. S2). Three patterns emerged. In the case of pv. *phaseolicola*, there was a strong (but transient) increase in *Aox1a* transcript at 12 h post-inoculation (Fig. 5a, Supporting Information Fig. S2) and this was accompanied by a dramatic increase in AOX protein, as measured at 24 h

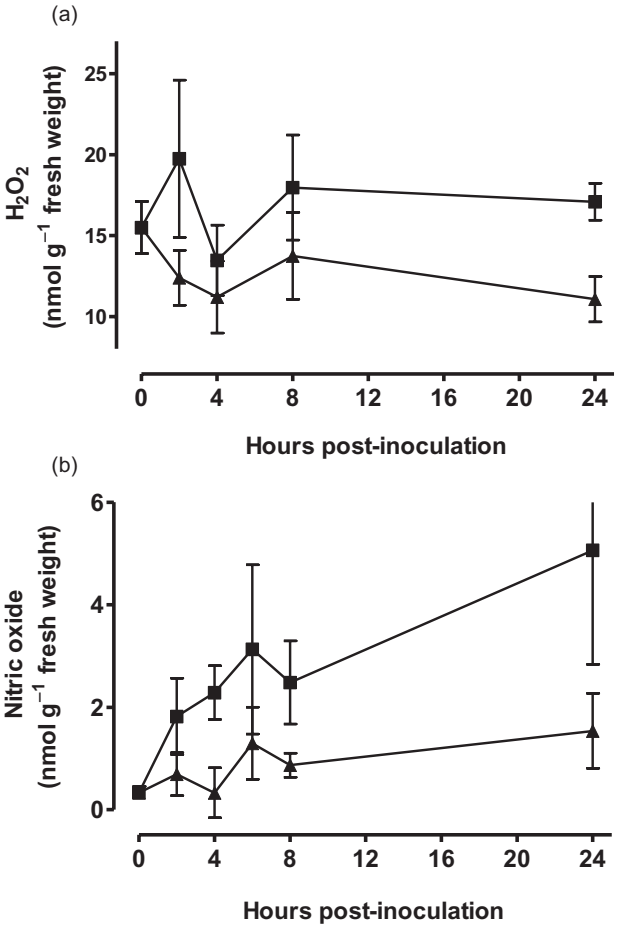


**Figure 4.** Levels of $H_2O_2$ (a) and NO (b) in tobacco leaf at different times post-inoculation with pathovars of *Pseudomonas syringae* (closed square, *maculicola*; closed triangle, *phaseolicola*). Data are the mean ± SE of four independent experiments, each of which showed similar results.

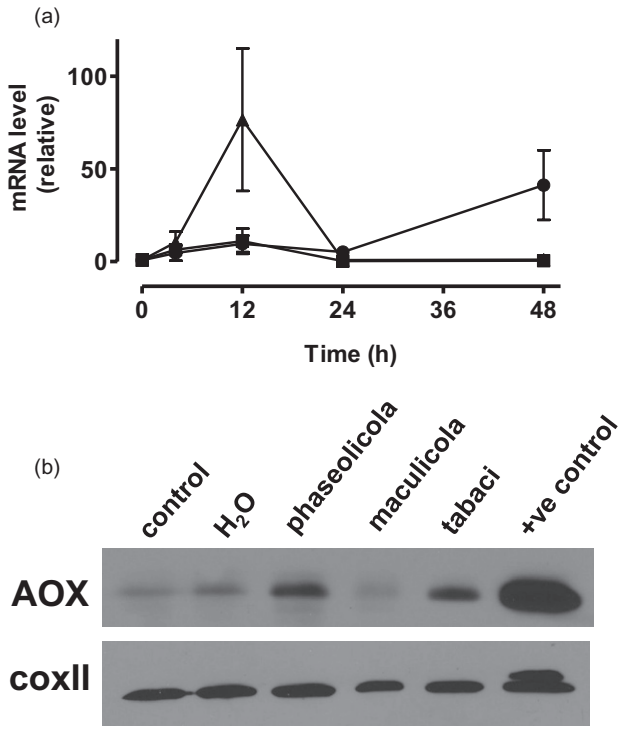


**Figure 5.** Level of *Aox1a* transcript (a) and alternative oxidase (AOX) protein (b) in tobacco leaf at different times post-inoculation with pathovars of *Pseudomonas syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). Data in (a) is the mean ± SE of densitometer analysis of Northern blots from three independent experiments normalized for diurnal changes in *Aox1a* transcript level, each of which showed similar results. For these data, the level of mRNA at each time point in mock-inoculated plants was arbitrarily set to 1 and used to normalize the data for plants inoculated with bacteria. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (not shown). For (b), mitochondria were isolated from tobacco leaf at 24 h after treatments. The mitochondrial proteins (100 $\mu$g) were then separated by SDS-PAGE, transferred to nitrocellulose and probed with an antibody against AOX. Confirmation of sample quality and equal loading between lanes was routinely checked by also examining levels of cyt oxidase subunit II (coxII) protein in the isolated mitochondria, as shown. The lane labelled $H_2O$ are leaves infiltrated with $H_2O$ while the lane labelled +ve control is a mitochondrial sample isolated from a transgenic tobacco line overexpressing AOX. Representative results are shown.

(Fig. 5b). However, in the case of the HR-inducing pv. *maculicola*, there was little if any change in *Aox1a* mRNA or protein in comparison to water-infiltrated controls. Similarly, pv. *tabaci* did not result in an early accumulation of *Aox1a* transcript, although a late accumulation at 48 h, when disease symptoms and necrotic cell death were already prevalent, was clearly evident. In this case, some increase in AOX protein was also already evident at 24 h.

For comparison, we examined AOX expression in *Arabidopsis* responding to *P. syringae* pv. *tomato*. In this case, both DC3000 (a virulent strain that caused necrotic cell death by 48 h) and DC3000 (*avrRpt2*) (an incompatible strain that induced the HR by 24 h) caused similar and modest induction of *Aox1a*, particularly at later stages (12–48 h) (Supporting Information Fig. S3). These results are generally similar to those found in a previous study (Simons *et al.* 1999). In the case of DC3000 (*avrRps4*) (an incompatible strain that did not induce HR), there was little change in *Aox1a* expression in comparison to control. We also examined the expression of other *Arabidopsis* AOX genes. A change in transcript level of *Aox1d* in response to each bacterial strain was similar to that seen with *Aox1a*, although the overall level of transcript was much less (data not shown). Levels of *Aox1c* and *Aox2* were not impacted by the treatments (data not shown).

## Other biotic stress-related genes are also differentially expressed in response to different *P. syringae* pathovars

Changes in tobacco *Aox1a* transcript were compared with that of other tobacco genes previously shown to respond to biotic stress. *HSR203J* and *HIN1* have each been previously judged as good marker genes for the HR response (Pontier *et al.* 1994; Gopalan, Wei & He 1996). Similarly, increased *PAL* gene expression is often associated with the HR (Vlot *et al.* 2009). These genes were all strongly induced within 4 h of inoculation with pv. *maculicola* (Fig. 6a–c). In the case of *HSR203J* and *HIN1*, there was also a later (12 h) induction by pv. *phaseolicola*. All three genes were only induced by pv. *tabaci* at a much later stage (24–48 h). *PR-1* is a well-known late-induced, SA-responsive defence gene to incompatible pathogens (Eulgem 2005). As expected, *PR-1* transcript did not change significantly until 24 h, at which time it was induced by the incompatible interactions (strongly by pv. *phaseolicola* and more moderately by pv. *maculicola*) (Fig. 6e). The compatible pv. *tabaci* did not induce *PR-1* through 48 h. *GRAS* likely encodes a transcription factor and its transcript level has previously been shown to respond to treatments such as antimycin A, NO, SA and $H_2O_2$, in a manner very similar to that of *Aox1a* (Maxwell, Nickels & McIntosh 2002). *GRAS* transcript was transiently induced (at 12 h) by pv. *phaseolicola*, not induced at all by pv. *maculicola*, and induced only late by pv. *tabaci* (Fig. 6f). These patterns mirror that seen with *Aox1a* transcript (Fig. 5a). Increased ethylene production is a common response to biotic stress (van Loon, Geraats & Linthorst 2006). The gene encoding ACC oxidase, which catalyses the last step in ethylene biosynthesis, increased transiently (at 12 h) in response to all these pathovars but most strikingly in response to pv. *maculicola* (Fig. 6d).

## Qualitatively similar changes in *Aox1a* transcript abundance can be seen in tobacco suspension cells treated with different concentrations of SA

Treatment of tobacco suspension cells with different concentrations of SA led to differences in the timing, extent

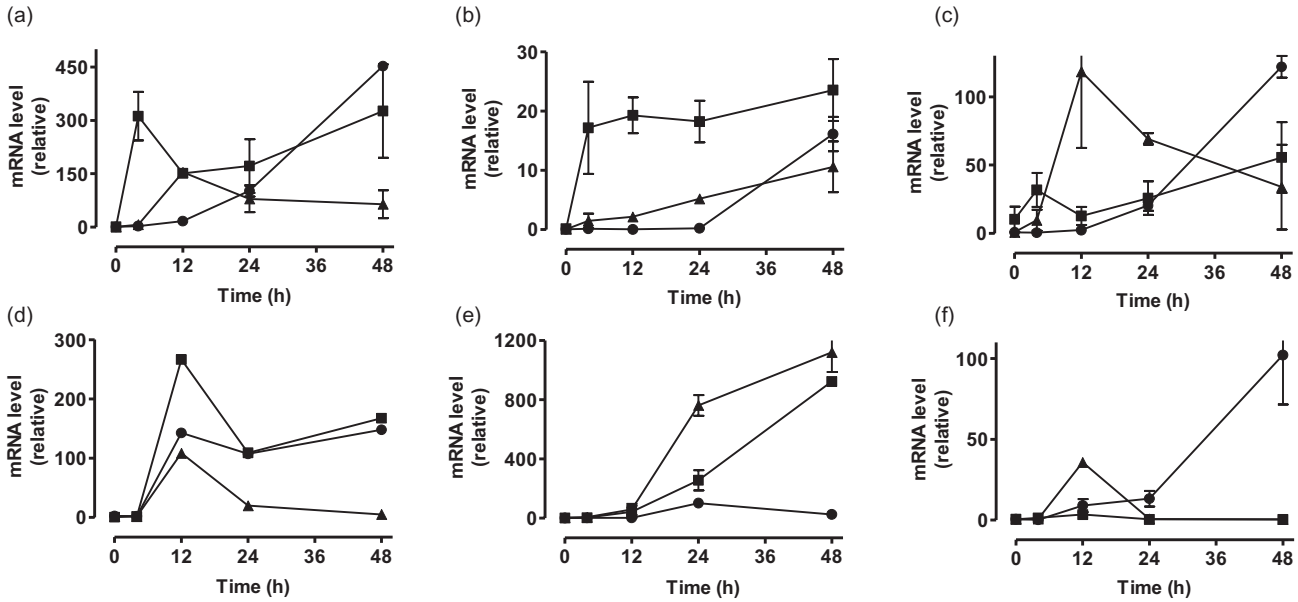


**Figure 6.** Level of *HSR203J* (a), *PAL* (b), *HIN1* (c), *ACC oxidase* (d), *PR-1* (e) and *GRAS* (f) transcript in tobacco leaf at different times post-inoculation with pathovars of *Pseudomonas syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). Data are the mean ± SE of densitometer analysis of Northern blots from two independent experiments, each of which showed similar results. For these data, the level of mRNA in the mock-inoculated plants has been arbitrarily set to 1 and used to normalize the data for plants inoculated with bacteria. Quality of RNA and confirmation of equal loading between lanes was routinely checked by ethidium bromide staining (not shown).

and type of cell death by the culture. It was therefore of interest to see what change in *Aox1a* gene expression accompanied these responses and whether these mirrored the changes seen during the response of tobacco leaf to the different bacterial pathovars.

A high concentration of SA (3 mM) led to rapid cell death, with complete death of the culture within 24 h (Fig. 7a). This was accompanied by a distinct pattern of breakdown of high-molecular-weight DNA to generate the low-molecular-weight 'DNA ladder' typical of PCD. As expected, this 'ladder' has bands of DNA corresponding in size to that of oligonucleosomal fragments (~180–200 bp) and multiples thereof (Fig. 7b). In contrast, treatment with a moderate concentration of SA (0.5 mM) led to a slower death of the culture, with only ~30% of cells dead by 24 h and ~85% by 48 h. This cell death was also accompanied by the breakdown of high-molecular-weight DNA but did not generate the DNA ladder typical of PCD, indicating that in this case, the death was necrotic. Low (0.1 mM) SA had little impact on cell viability (Fig. 7a) and cultures maintained high-molecular-weight DNA through 120 h (Fig. 7b).

The SA treatments led to distinct changes in *Aox1a* gene expression. With the treatment that did not induce significant cell death (0.1 mM SA), there was a rapid (within 2 h), large and transient induction of *Aox1a* transcript (Fig. 7c,d). With the treatment generating PCD (3.0 mM SA), there was no induction, but rather a slight decline in *Aox1a* transcript. With the treatment causing necrotic cell death (0.5 mM), there was also no early induction by 2 h but rather an induction in the longer term.

## SOD enzymes show distinct changes in activity in response to different *P. syringae* pathovars

The major SOD enzymes (MnSOD, CuZnSOD, FeSOD) were identified on activity gels according to their sensitivity to different inhibitors. The gels were then used to monitor the activity of each enzyme over a 48 h period following inoculation of tobacco leaf. All three enzymes, but particularly CuZnSOD, increased in activity by 24 h, and further by 48 h, following inoculation with pv. *phaseolicola* (Fig. 8). In the case of pv. *tabaci*, none of the activities changed by 24 h. A small increase in MnSOD and CuZnSOD activity did appear, however, by 48 h, with still no change in FeSOD. In response to pv. *maculicola*, the activity of CuZnSOD and FeSOD perhaps increased over the 48 h period, while MnSOD clearly declined by 24 h and further by 48 h. In summary, the activities of all three enzymes tended to increase or remain unchanged in response to all three pathovars, with the exception of MnSOD activity following inoculation with the HR-inducing pv. *maculicola*, in which case, activity declined dramatically (Fig. 8).

## Dynamic changes in mitochondrial $O_2^-$ level in response to different *P. syringae* pathovars

Mitochondrial $O_2^-$ level in tobacco leaf mesophyll cells was assessed by using confocal microscopy to image MitoSOX Red fluorescence. To ensure no confounding effects due to autofluorescence, images of unlabelled tissue were obtained using the acquisition settings and longest exposure time

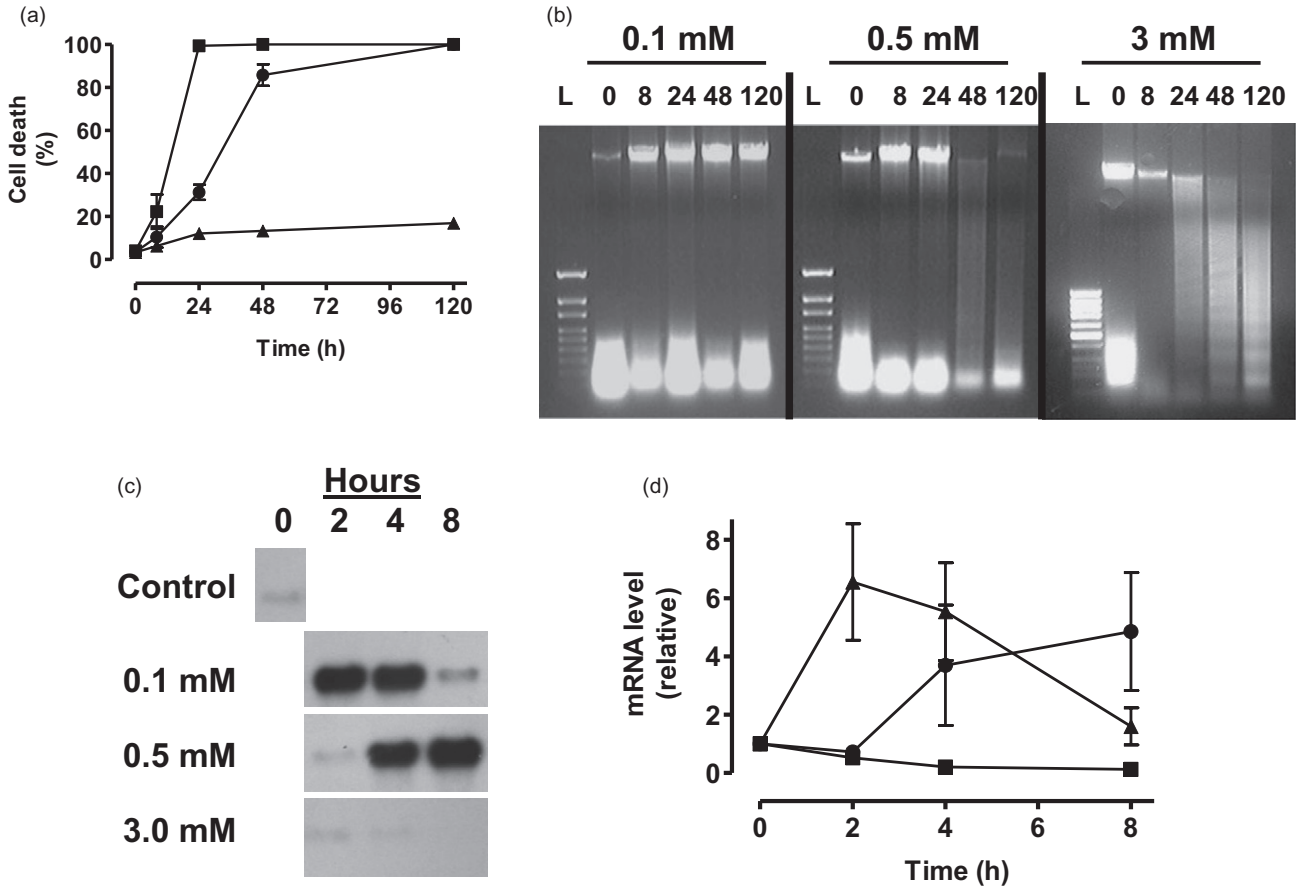


**Figure 7.** Effects of SA on tobacco suspension cells. (a) Viability of suspension cell cultures following treatment with 0.1 mM (triangle), 0.5 mM (circle) or 3 mM (square) salicylic acid (SA). Data are the mean ± SE of two independent experiments. Note that in some cases, error bars are smaller that the data symbol. (b) Representative agarose gel analysis of DNA extracted from suspension cells at different times after treatment with 0.1, 0.5 or 3.0 mM SA. The lane marked L is a 100 bp low-molecular-weight DNA ladder, but please note that the commercial ladder corresponding with the 3 mM lanes differs from that corresponding with the 0.1 and 0.5 mM lanes. The estimated size of the low-molecular-weight bands of DNA seen to accumulate in the 3 mM (48 h) and 3 mM (120 h) lanes are ~170, 350, 550 and 770 bp. (c) Representative Northern blot showing changes in the level of *Aox1a* transcript in suspension cells at different times after treatment with 0.1, 0.5 or 3.0 mM SA. (d) Level of *Aox1a* transcript in suspension cells at different times after treatment with 0.1 mM (triangle), 0.5 mM (circle) or 3.0 mM (square) SA. Data are the mean ± SE of densitometer analysis of Northern blots from three independent experiments, each of which showed similar results. Note that the level of *Aox1a* in control (untreated) cells (time 0 h) was arbitrarily set at 1.

being used for labelled samples. Chlorophyll autofluorescence was detected through a 650 nm long-pass filter, but no signal was detected in the 585–615 nm range. As another control, leaf segments with the lower epidermis removed were floated on solution containing the Complex III inhibitor antimycin A (10 $\mu$m, 1 h, RT) prior to loading with MitoSOX Red and, as expected, this dramatically elevated MitoSOX Red fluorescence. There are reports that MitoSOX Red can cause mitochondrial rupture and signal relocation to the nucleus at higher concentration (Robinson *et al*. 2008) and we did observe labelling of nuclei. However, we observed no visible difference in the number of mitochondria in leaf labelled with MitoSOX Red compared to Mitotracker Red (data not shown).

In leaves inoculated with the HR-inducing pv. *maculicola*, high levels of $O_2^-$ were clearly evident by 4 h post-inoculation, the earliest time point examined, and persisted through 24 h (Fig. 9). In contrast, there was little change in $O_2^-$ level, compared to mock-inoculated leaves, at any time point following inoculation with pv. *phaseolicola*. Finally, pv. *tabaci* induced only a late (24 h) increase in $O_2^-$ but not the early mitochondrial $O_2^-$ burst seen in response to pv. *maculicola* (Fig. 9).

## DISCUSSION

Research suggests that the mitochondrion can be an important intracellular source of ROS during plant PCD, generating a so-called mitochondrial oxidative burst. Since AOX is an important regulator of ROS generation by the ETC, several studies have examined the relationship between AOX and susceptibility to PCD induced by various biotic or abiotic stimuli. For example, overexpression of AOX in transgenic *Arabidopsis* protoplasts was found to protect

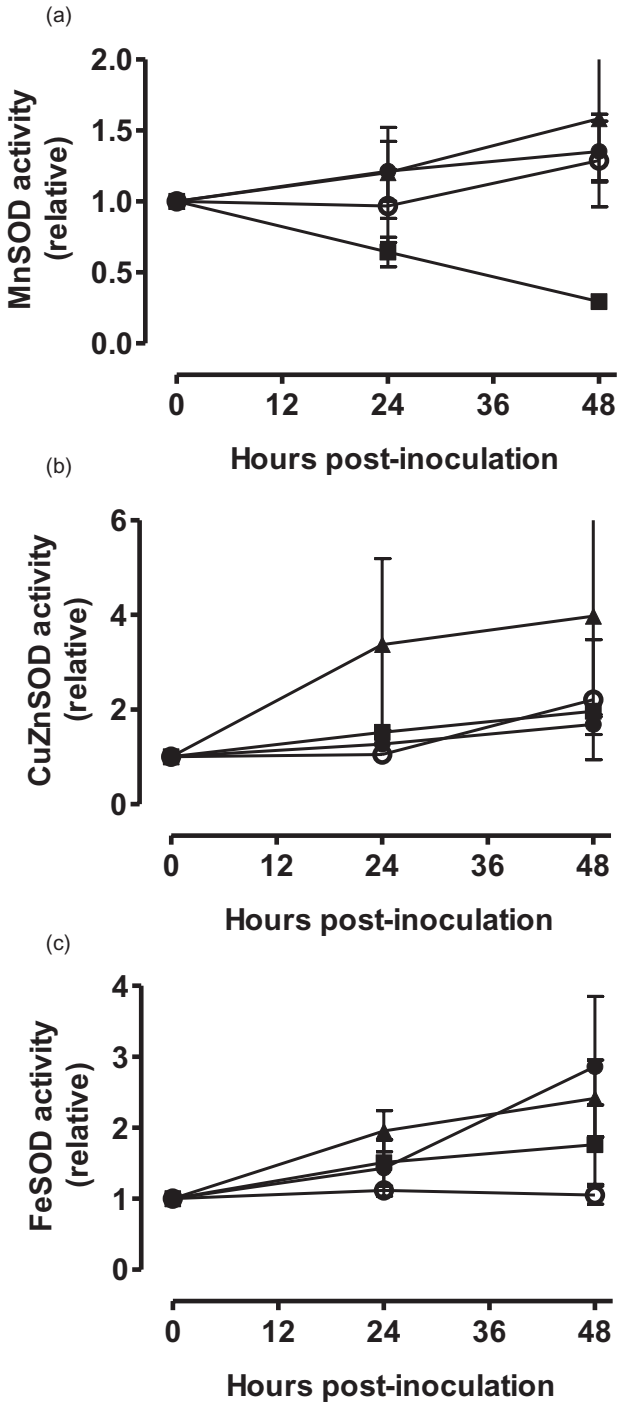


**Figure 8.** Superoxide dismutase activity in tobacco leaf at different times post-mock inoculation with $H_2O$ (open circle) or post-inoculation with pathovars of *Pseudomonas syringae* (closed circle, *tabaci*; closed square, *maculicola*; closed triangle, *phaseolicola*). An in-gel activity assay was used to separate the activity of MnSOD (a), CuZnSOD (b) and FeSOD (c). Data are the mean ± SE of three independent experiments, each of which showed similar results. Note that in some cases, error bars are smaller that the data symbol.

against aluminium-induced PCD (Li & Xing 2011), while overexpression in tobacco resulted in slightly smaller tobacco mosaic virus-induced HR lesions (Ordog, Higgins & Vanlerberghe 2002). Conversely, suppression of AOX in transgenic tobacco suspension cells increased their susceptibility to SA or $H_2O_2$-induced PCD (Robson & Vanlerberghe 2002; Amirsadeghi *et al.* 2006). Collectively, such studies suggest that AOX may provide a level of protection against PCD. Given this, we postulated that during natural examples of PCD that are likely of benefit to the plant (such as the HR), AOX (with its potential to act as a negative regulator of PCD) would not be induced, or might even decline, such as to promote the response. To test this, we compared responses to three different *P. syringae* pathovars. The differential responses to these pathovars was first characterized by examining the kinetics of cell death, changes in key biotic stress signalling molecules and changes in established biotic stress-related genes. These analyses established three distinct plant responses to the pathovars: a compatible response that did not elicit typical defences, such as increased SA, and which was associated, at a late stage, with disease-associated necrotic cell death (pv. *tabaci*); an incompatible response that included well-known defence responses, such as increased SA and *PR-1* transcript, but did not include any HR cell death (pv. *phaseolicola*); and an incompatible response that included strong defence responses (such as rapid accumulation of SA, NO and $H_2O_2$, and induction of HR-associated genes) and the HR, which was then able to restrict any further proliferation of the pathogen (pv. *maculicola*).

### During an HR-inducing interaction, AOX expression is not elevated despite a strong increase of potentially inductive signalling molecules

The signalling molecules SA, NO and $H_2O_2$ have each been shown to strongly induce AOX in numerous species and contexts, and these molecules all showed an early and strong accumulation in response to the HR-inducing pv. *maculicola*. In addition, the strong induction of ACC oxidase gene expression is an indirect evidence that ethylene was likely also being produced, as has previously been documented in the HR response of tobacco to *P. syringae* (Mur *et al.* 2008). Ethylene is strongly implicated in the induction of AOX by abiotic stress (Ederli *et al.* 2006). However, despite the increase of these signalling molecules, there was no increase in *Aox1a* transcript or AOX protein in response to pv. *maculicola*. Hence, during the HR, AOX expression was uncoupled from several of the signalling molecules thought to otherwise promote its induction. In contrast, pv. *phaseolicola* (which induced defence responses, but in the absence of a HR) did elicit a strong transient increase in *Aox1a* transcript, accompanied by increased AOX protein. In the case of pv. *tabaci*, AOX expression was induced but only much later after infection, just prior to necrotic death of the diseased tissue.

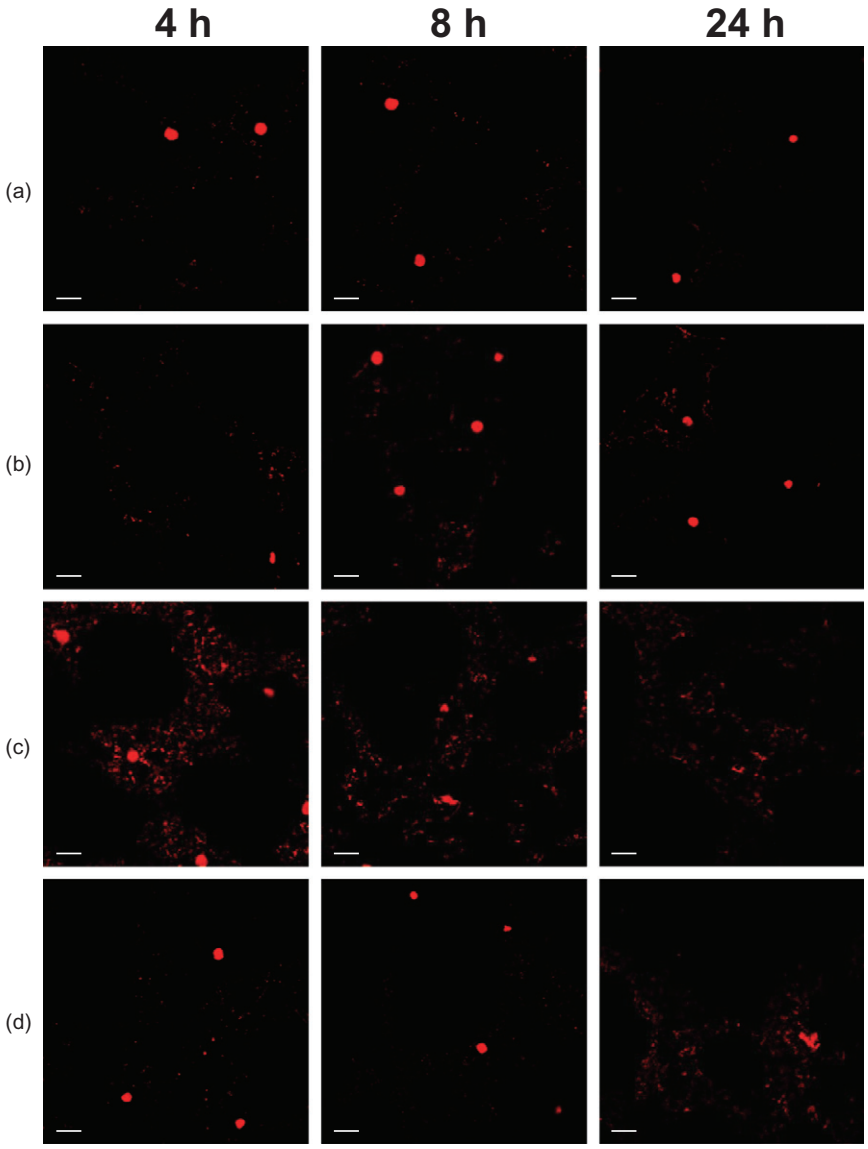


**Figure 9.** Laser-scanning confocal microscope images of mitochondrial $O_2^-$ in mesophyll cells at different times post-mock inoculation with $H_2O$ (a) or post-inoculation with pathovars of *Pseudomonas syringae*: pv. *phaseolicola* (b), pv. *maculicola* (c) and pv. *tabaci* (d). All images are maximum intensity projections of Z-series (8–16 $\mu$m in depth) and are representative results from four independent experiments, each of which showed similar results. Scale bar = 20 $\mu$m.

Our results with tobacco differ from a previous study in *Arabidopsis* (Simons *et al.* 1999). It showed that both a compatible and an HR-inducing incompatible *P. syringae* induced *Aox1a*, but with the earliest and strongest induction occurring during the HR. In that study, *Aox1a* was induced strongly by 6 and 22 h after infection with incompatible bacteria, and the authors state that HR tissue collapse occurred by 24 h, although the cell death was not characterized any further. We also observed an increase in *Arabidopsis Aox1a* transcript by both compatible strain DC3000 and HR-inducing strain DC3000 (*avrRpt2*). Furthermore, *Aox1a* was not induced during the incompatible response to DC3000 (*avrRps4*) that lacked the HR. However, the absolute changes in *Aox1a t*ranscript that we observed in *Arabidopsis* were much more subtle than we would see, for example, in tobacco responding to pv. *phaseolicola*. Lacomme & Roby (1999) also identified *Aox1a* as a gene induced in *Arabidopsis* suspension cells by an avirulent, but not virulent, *Xanthomonas campestris* and suggested it as an early marker of the HR. However, it is unclear from that study whether the treatment of suspension cells with the avirulent bacteria was actually inducing cell death. Studies in citrus and lettuce leaf have also noted AOX induction in response to HR-inducing bacteria (Kiba *et al.* 2008; Daurelio *et al.* 2009), while harpin, an HR-inducing bacterial elicitor, increased *Aox1a* expression

in *Arabidopsis* suspension cells (Krause & Durner 2004) and AOX activity in *Nicotiana sylvestris* (Vidal *et al*. 2007). Similar to our findings, a few other reports have suggested that AOX is in fact not induced during a PCD response. In tobacco cells challenged by metabolic products of a fungal pathogen that induced PCD, it was noted that AOX capacity was reduced (Cheng *et al*. 2011). However, total respiration was also strongly reduced and AOX transcript and protein were not examined, hence making a complete interpretation of those results difficult. Similarly, AOX was not induced by harpin in *N. sylvestris* leaf (Boccara *et al*. 2001). Overall, the contrasting results suggest that there may be fundamental differences between species regarding the role of AOX in cell death programs, factors regulating AOX gene expression or baseline AOX level between species. Some differences between studies may also be due to differences in experimentation and interpretation. Our study had the advantage of being able to directly compare multiple distinct responses to different bacterial pathovars, accompanied by direct measures of cell death, changes in signalling molecules (SA, NO, $H_2O_2$), changes in mitochondrial $O_2^-$ and careful characterization of *Aox1a* against background diurnal changes in expression, diurnal changes that have also been previously reported in tobacco (Dutilleul *et al*. 2003). In addition, our studies have been done *in planta* (rather than suspension cells where oxidative poise may differ; Halliwell 2003), and in response to bacteria rather than an HR-inducing elicitor, that might not 'elicit' the same or complete plant response as that of live bacteria (Mur, Kenton & Draper 2005).

At present, we do not know the mechanism by which *Aox1a* expression is suppressed during the HR, despite the dramatically increased level of potential inductive signalling molecules. A clue to this might be provided by the experiment on suspension cells in which it was clear that while low concentrations of SA were highly effective at inducing *Aox1a*, higher concentrations of SA had no effect. This suggests that either high concentrations of SA itself or some downstream consequence of high SA triggers a suppression rather than induction of *Aox1a* expression. Significantly, the cell results mirror well the relationship between *Aox1a* expression, SA level and cell fate seen in leaf during the incompatible interactions. The HR-inducing pv. *maculicola* showed the strongest early induction of SA, lacked any *Aox1a* induction and resulted in PCD. Alternatively, pv. *phaseolicola* showed less early accumulation of SA, a strong transient *Aox1a* induction and no PCD.

Similar to our results, several studies have noted that AOX expression can be strongly induced at the late stages of compatible bacterial interactions that culminate in a disease-associated cell death, or other cases of necrotic cell death. Examples are reported in *Arabidopsis* (Simons *et al*. 1999), lettuce (Kiba *et al*. 2009) and wheat (Sugie, Murai & Takumi 2007). The late AOX induction may represent an extreme response to a collapse of energy metabolism or a non-specific response to cellular dysfunction.

## Do AOX and MnSOD act in a coordinated manner to define the strength and specificity of a ROS signal from the mitochondrion?

Our results indicate that MnSOD activity declines in tobacco leaf in response to the HR-inducing pv. *maculicola*. This was distinct from the response of MnSOD activity to the other pathovars, as well as from the response of the other SOD enzymes, which tended to either increase or remain unchanged. Particularly, the increase in CuZnSOD is consistent with previous studies (Kliebenstein *et al*. 1999). However, to our knowledge, few studies have examined changes in MnSOD activity during PCD or in response to biotic stress. A microarray analysis of senescence in *Arabidopsis* suspension cells did report a decrease in both AOX and MnSOD transcript, although heat shock-induced PCD had the opposite effect on both genes (Swidzinski, Sweetlove & Leaver 2002). Also interesting in this respect is a *N. sylvestris* cytoplasmic male-sterile mutant (CMSII) that exhibits both a higher constitutive level of AOX and MnSOD, and a delayed senescence phenotype (Dutilleul *et al*. 2003). It was also shown that PCD of barley aleurone cells is preceded by a loss of SOD activity, with MnSOD appearing to be the first to decline (Fath, Bethke & Jones 2001).

At present, we do not know the mechanism responsible for loss of MnSOD activity in response to pv. *maculicola*. A previous study showed that loss of MnSOD activity during fruit senescence correlated with carbonylation of the protein (Qin *et al*. 2009). Interestingly, human MnSOD is inactivated by $ONOO^-$ via tyrosine nitration (Castro *et al*. 2011). Recently, $ONOO^-$, which is formed by reaction of NO with $O_2^-$, has been shown to accumulate during the HR (Gaupels *et al*. 2011).

There are also means by which the low constitutive level of AOX protein that still exists in tobacco leaf after pv. *maculicola* infection could be inactivated. For example, a high level of $H_2O_2$ has the potential to convert AOX to its oxidized low activity form (Vanlerberghe, Yip & Parsons 1999). AOX activity is also sensitive to the lipid peroxidation product 4-hydroxy-2-nonenal (Winger, Millar & Day 2005) and, interestingly, was identified as a target of tyrosine nitration in sunflower hypocotyls (Chaki *et al*. 2009).

A major finding of our study is that the HR of tobacco leaf is preceded by an early and persistent mitochondrial burst of $O_2^-$. Studies have shown that ROS are important signalling molecules, and that the type of ROS (e.g., $O_2^-$ versus $H_2O_2$), the level of ROS and the location of the ROS will impact the cellular programs that they elicit (Apel & Hirt 2004; Foyer & Noctor 2005; Mittler *et al*. 2011). We suggest that AOX and MnSOD are uniquely positioned to impact both the strength and specificity of ROS signals in the mitochondrion (Fig. 10). AOX will impact the *strength* of the ROS signal by modulating the rate of $O_2^-$ generation by the ETC. Then, the activity of MnSOD (the sole enzymatic means to scavenge matrix $O_2^-$ and convert it to $H_2O_2$) will determine the *specificity* of the signal by determining the relative levels of $O_2^-$ and $H_2O_2$ in the matrix. In

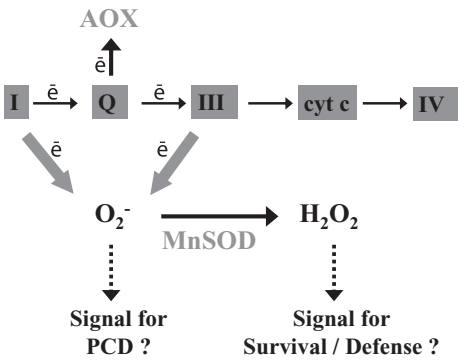


**Figure 10.** Reactive oxygen species (ROS) are important signalling molecules and the type of ROS (e.g., $O_2^-$ versus $H_2O_2$), level of ROS and location of ROS impact the cellular programs that they elicit. The model outlined here suggests that alternative oxidase (AOX) and MnSOD are uniquely positioned to impact both the strength and specificity of ROS signals in the mitochondrion. AOX will impact the *strength* of the ROS signal by modulating the rate of $O_2^-$ generation by the mitochondrial ETC. Then, the activity of MnSOD (the sole enzymatic means to scavenge matrix $O_2^-$ and convert it $H_2O_2$) will determine the *specificity* of the signal by determining the relative levels of $O_2^-$ and $H_2O_2$ in the matrix. Since $H_2O_2$ is membrane-permeable, while $O_2^-$ is not, MnSOD may also influence the distribution of mitochondrial-generated ROS between mitochondrion and cytosol. We propose that the suppression of both AOX and MnSOD seen in this study during the HR of tobacco to *Pseudomonas syringae* pv. *maculicola* is important to generating the early and persistent mitochondrial $O_2^-$ burst that was observed. More generally, a coordinated activity of AOX and MnSOD represents a potential means to construct multiple distinct mitochondrial ROS signatures that could lead to distinct cellular responses.

addition, since $H_2O_2$ is membrane permeable, while $O_2^-$ is not, MnSOD may also influence the distribution of mitochondrial-generated ROS between the mitochondrion and the cytosol (Fig. 10).

Numerous studies have shown a suppression of different ROS-scavenging systems during the HR and other forms of PCD, and it has been suggested that this aspect of the cellular response is necessary to allow a sufficient accumulation of ROS needed to promote the PCD (Foyer & Noctor 2005; Vlot *et al*. 2009). Most studies to date have examined suppression of the ROS-scavenging systems of the cytosol, chloroplast and peroxisome, relating this then to ROS sources such as NADPH oxidase, photosynthesis and photorespiration. Consistent with the theme, we suggest that coordinated suppression of AOX and MnSOD is important in defining a mitochondrial $O_2^-$ burst during the HR.

## Is mitochondrial $O_2^-$ an important signal for the HR?

Our results show that mitochondrial $O_2^-$ increases dramatically during the response of tobacco to the HR-inducing pv. *maculicola* and suggest that this response is the result of a coordinated suppression of AOX and MnSOD. This leads to the important question of whether mitochondrial $O_2^-$ is in fact an important signal for the HR. While it is generally accepted that $H_2O_2$, $O_2^-$, NO and likely other reactive nitrogen species are important players in the initiation and execution of the HR and other defence responses, the detail of their roles requires further investigation. The *Arabidopsis* spontaneous cell death mutant *lsd1* has highlighted the specific importance of $O_2^-$, particularly when in combination with SA, to promote PCD (Mazel & Levine 2001; Epple *et al*. 2003). Unlike wild-type, *lsd1* does not induce CuZnSOD in response to SA, and the increased $O_2^-$ increases their susceptibility to SA-induced PCD.

One hypothetical means by which mitochondrial $O_2^-$ might be acting mechanistically to promote PCD is via its reaction with NO to produce $ONOO^-$. $ONOO^-$ could impact the function of proteins via the nitration of key tyrosine residues and studies have just begun to identify those proteins subject to tyrosine nitration during the HR (Cecconi *et al*. 2009). Alternatively, some of the pro-survival functions described for NO (Yun *et al*. 2011) might be attenuated if NO is being scavenged by $O_2^-$. Similarly, some of the pro-survival functions described for $H_2O_2$ (Torres, Jones & Dangl 2006) might be attenuated in the absence of conversion of mitochondrial $O_2^-$ to $H_2O_2$. We previously suggested that complex III-derived $O_2^-$ could induce PCD in tobacco suspension cells, based on the disparate susceptibility of cells to two complex III inhibitors with differential capacities to generate $O_2^-$ (Robson, Zhao & Vanlerberghe 2008). Elucidating the functional importance of the mitochondrial $O_2^-$ burst clearly represents a rich area for further study.

While we have emphasized the importance of a mitochondrial $O_2^-$ burst as a potential pro-death signal in support of the HR, it is worth noting that the strong induction of AOX, and to a lesser extent MnSOD, during the response to pv. *phaseolicola* might also be of importance. In this case, the lower overall mitochondrial-generated ROS, along with perhaps a shift toward $H_2O_2$, at the expense of $O_2^-$, might be an important pro-survival signal to prevent HR and/or induce other defence gene expression. Hence, we view the coordinated activity of AOX and MnSOD as a potential means to construct multiple distinct mitochondrial ROS signatures that could lead to distinct cellular responses (Fig. 10).

## ACKNOWLEDGMENTS

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Diurnal changes in the level of *Aox1a* and *PAL* transcript in tobacco leaf either left untreated or mock-inoculated with $H_2O$.

**Figure S2.** Representative Northern blot showing level of *Aox1a* transcript in tobacco leaf at different times post-inoculation with pathovars of *P. syringae* or after mock-inoculation with $H_2O$.

**Figure S3.** Level of *Aox1a* transcript in *Arabidopsis* rosette leaves at different times post-inoculation with strains of *P. syringae* pv. *tomato* or after mock-inoculation with $MgCl_2$.

**Table S1.** Primer sequences used for RT-PCR.

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