Systemic Induction and Role of Mitochondrial Alternative Oxidase and Nitric Oxide in a Compatible Tomato–*Tobacco mosaic virus* Interaction

Li-Jun Fu,¹ Kai Shi,¹ Min Gu,¹ Yan-Hong Zhou,¹ De-Kun Dong,³ Wu-Sheng Liang,⁴ Feng-Ming Song,⁴ and Jing-Quan Yu^{1,2}

¹Department of Horticulture, Huajiachi Campus, Zhejiang University, ²Key Laboratory of Horticultural Plants Growth, Development and Quality Improvement, Agricultural Ministry of China, ³The Institute of Crop and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences, and ⁴Department of Plant Protection, Institute of Biotechnology, Huajiachi Campus, Zhejiang University, Kaixuan Road 268, Hangzhou, P.R. China

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The role of mitochondrial alternative oxidase (AOX) and the relationship between AOX and nitric oxide (NO) in virus-induced systemic defense to *Tobacco mosaic virus* (TMV) were investigated in susceptible tomato (*Solanum lycopersicum*) plants. TMV inoculation to the lower leaves induced a rapid NO synthesis and AOX activation in upper uninoculated leaves as early as 0.5 day postinoculation. Application of exogenous potassium cyanide (KCN, a cytochrome pathway inhibitor) at nonlethal concentrations and NO donor diethylamine NONOate (DEA/NO) to the upper uninoculated leaves greatly induced accumulation of AOX transcript, reduced TMV viral RNA accumulation, and increased the leaf photochemical quantum yield at photosystem II. Pretreatment with NO scavenger almost completely blocked TMV-induced AOX induction and substantially increased TMV susceptibility. Salicylhydroxamic acid (SHAM, an AOX inhibitor) pretreatment reduced the DEA/NO-induced cyanide-resistant respiration and partially compromised induced resistance to TMV. Conversely, KCN and SHAM pretreatment had very little effect on generation of NO, and pretreatment with NO scavenger did not affect KCN-induced AOX induction and TMV resistance. These results suggest that TMV-induced NO generation acts upstream and mediates AOX induction which, in turn, induces mitochondrial alternative electron transport and triggers systemic basal defense against the viral pathogen.

Mitochondrial respiration provides energy necessary to drive metabolic and transport processes in cells. Plant mitochondria possess two different pathways of electron transport at the ubiquinone level: the cytochrome pathway (Cyt pathway) terminated at cytochrome *c* oxidase (COX) and the cyanide (CN)-resistant alternative pathway which involves only a single quinol oxidase termed the alternative oxidase (AOX) (Siedow and Umbach 1995; Arnholdt-Schmitt et al. 2006). Electron transfer through the Cyt pathway is coupled to the generation of an electrochemical gradient used to produce ATP. However, electron flow from ubiquinone to AOX is not coupled to ATP production. The function or functions of this nonphosphorylating path in nonthermogenic respiratory metabolism is not well defined (Purvis and Shewfelt 1993;

Amirsadeghi et al. 2007; Vidal et al. 2007). Although AOX protein level and CN-resistant respiration are low in unstressed plants, they were all enhanced after exposure to pathogens, low temperature, hypoxia, high light intensity, and other stresses (Szal et al. 2003; Yoshida et al. 2007; Király et al. 2008; Watanabe et al. 2008). Therefore, the CN-resistant alternative pathway may play an important role in stress tolerance. It has also been suggested that enhanced operation of the alternative pathway plays a role in protecting plant cells from the attack of reactive oxygen species (ROS) (Maxwell et al. 1999; Umbach et al. 2005) and in the maintenance of plant homeostasis in response to biotic and abiotic stresses (Moore et al. 2002).

Plant mitochondria are implicated in both local and systemic defenses against pathogen infection. In *Arabidopsis*, induction of AOX in leaves infiltrated with both avirulent and virulent *Pseudomonas syringae* pv. *tomato* DC 3000 are accompanied with local necrosis, while increased respiration appears to be associated with symptom development rather than resistance response (Simons et al. 1999). Treatment with a proteinaceous bacterial elicitor, harpin, also resulted in an increased expression of the *AOX1a* gene in *Arabidopsis* suspension cells, and the induction of AOX was suggested to be a protecting or scavenging system (Krause and Durner 2004). Nevertheless, the significance of the alternative respiratory pathway during pathogen attack still remains to be elucidated.

Unlike some fungi and eubacteria, viruses, which do not have AOX protein (Vanlerberghe and McIntosh 1997; Avila-Adame and Köller 2002), provide good pathogen systems for investigating the role of cyanide-resistant respiration in the plant defense response. AOX is often induced during plant–virus interactions (Lennon et al. 1997; Chivasa and Carr 1998). In directly inoculated tissue, several lines of evidence have supported the idea that the AOX-dependent signal transduction pathway plays roles in mediating virus localization and limiting host tissue necrosis (Chivasa and Carr 1998; Dutilleul et al. 2003; Love et al. 2005; Király et al. 2008). However, the role of AOX in plant systemic basal defense against viral pathogens remains elusive. *NPR1* is a key regulator of systemic defense responses in both compatible or incompatible plant interactions with bacterial and fungal pathogens (Chivasa and Carr 1998; Wong et al. 2002) but not with viral pathogens. Tobacco resistance to *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), and *Cucumber mosaic virus* (CMV) can be stimulated by inhibitors of the Cyt path-

Corresponding author: Kai Shi; Telephone: 0086-57186971120; Fax: 0086-57186049815; E-mail: kaishi@zju.edu.cn

way (e.g., antimycin A and potassium cyanide [KCN]) but the resistance is not associated with NPR1-regulated *PR-1* expression. Similarly, virus replication and systemic movement in tobacco were antagonized by the AOX inhibitor salicylhydroxamic acid (SHAM) (Chivasa et al. 1997; Naylor et al. 1998; Mayers et al. 2005). In *Arabidopsis thaliana*, resistance to *Turnip vein clearing virus* (TVCV) can be induced by cyanide and antimycin A in *npr1* mutants (Kachroo et al. 2000; Wong et al. 2002). These observations suggest that AOX may play a crucial role in the induction of systemic basal defense to viruses through a signaling pathway distinct from those against bacterial and fungal pathogens (Murphy et al. 1999; Wong et al. 2002; Singh et al. 2004). However, results from these experiments should be interpreted with caution because these results are mainly obtained with tobacco and *Arabidopsis*, and some of these experiments were carried out using detached leaves (Chivasa et al. 1997; Chivasa and Carr 1998). In squash (*Cucurbita pepo*), neither antimycin A or KCN induced resistance to CMV, and AOX inhibitors that compromise salicylic acid (SA)-induced resistance to CMV in tobacco did not inhibit SA-induced resistance to the virus in squash (Mayers et al. 2005). In *Phaseolus vulgaris*, AOX genes showed low constitutive expression after infection by *White clover mosaic virus* (WCIMV) (Gális et al. 2004). To date, it is not clear whether AOXs in upper uninoculated leaves play roles in systemic basal defense in compatible plant-virus interactions.

In recent years, nitric oxide (NO) has been demonstrated to be an important signaling molecule that regulates plant development and responses to environmental cues, including pathogen infection (Floryszak-Wieczorek et al. 2007; Asai et al. 2008; Tada et al. 2008). Huang and associates (2002) demonstrated the NO-induced transcriptional activation of AOX and the CN-resistant respiration in *Arabidopsis* suspension cells. Inversely, several lines of evidence have indicated that mitochondria may be one of the main sites of NO synthesis (Salgado et al. 2006). Mitochondrial electron transport was found to be involved in NO emission from tobacco leaves and cell suspensions (Planchet et al. 2005). These studies indicate that NO generation may be connected with AOX induction in plants. However, the interrelationship between mitochondrial AOX induction and NO generation as well as their roles in plant antiviral response remains to be determined.

Like other important crop plants, tomato can be infected by a large number of virulent viral pathogens. In the present study, using pharmacological and biochemical approaches, we have investigated the role of AOX and the relationship between AOX and NO in compatible interactions between tomato and TMV (U1 strain). TMV-induced AOX induction is enhanced by NO and reversed by NO synthesis inhibition, whereas KCN and SHAM have no significant effect on TMV-induced NO evolution. Our results show that NO is required for TMV-induced AOX induction which, in turn, induces mitochondrial alternative electron transport and promotes systemic basal defense against TMV.

RESULTS

Systemic induction of NO accumulation and CN-resistant respiration by TMV infection.

CN-resistant respiration and NO accumulation were determined in the compatible interactions between TMV and tomato plants (Figs. 1 and 2). In upper uninoculated leaves, CN-resistant respiration started to increase from 0.5 day postinoculation (dpi) and reached a maximum at 1 dpi. Meanwhile, there was significant increase in total respiration from 1 to 7 dpi (Fig. 1A). The relative transcript abundance of AOX genes at 4 dpi was analyzed by real-time reverse-transcription polymerase chain reaction (RT-PCR) (Fig. 1B). TMV inoculation led to 7.1-, 2.9-, and 2.6-fold increases in expression of *AOX1a*, *AOX1b*, and *AOX1c*, respectively, but had little effect on the *AOX2* transcript. Therefore, the *AOX1a*, *AOX1b*, and *AOX1c* genes were selected for further study.

Concomitant with the induction of CN-resistant respiration, NO generation was increased in upper uninoculated leaves by 3.5-fold at 0.5 dpi (Fig. 2) and there was less increase afterward. In TMV-inoculated plants, NO content was 50.8% higher than that in mock-inoculated plants at 10 dpi.

TMV-induced AOX induction is enhanced by NO and reversed by NO synthesis inhibition but KCN and SHAM did not influence TMV-induced NO evolution.

To investigate the possible involvement of mitochondrial AOX and NO in response to TMV inoculation, we first analyzed TMV-induced CN-resistant respiration and AOX tran-

Fig. 1. Changes in leaf respiratory rate and transcript level of alternative oxidase (AOX) genes in upper uninoculated leaves of tomato plants postinoculation with *Tobacco mosaic virus* (TMV) on a lower leaf. **A**, Time-course changes in the leaf total respiration rate and cyanide (CN)-resistant respiration rate. Squares, total respiration; circles, CN-resistant respiration. Open symbols indicate mock plants and closed symbols indicate TMV-inoculated plants. **B**, Transcript level of AOX genes in mock-inoculated (white bars) and TMV-inoculated (gray bars) plants were measured by quantitative real-time polymerase chain reaction. Leaf samples were collected 4 days postinoculation. Values are given after normalization with *Actin* and compared with the mock-inoculated plants (set at 1). Results are mean values $\pm$ standard deviation; $n = 4$. Different letters depict significant differences between the treatments ($P < 0.05$).

scripts as affected by exogenous NO (Fig. 3). Pretreatment with diethylamine NONOate (DEA/NO), a NO chemical donor, increased TMV-induced CN-resistant respiration and *AOX1a*, *AOX1b*, and *AOX1c* transcripts by 24.2, 9.8, 56.3, and 109.5%, respectively, over those in the unpretreated plants. In contrast, treatment with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, a specific NO scavenger) completely blocked the induction of CN-resistant respiration and AOX transcripts. As expected, the TMV-induced increase in CN-resistant respiration and transcripts of *AOX1a*, *AOX1b*, and *AOX1c* genes were further enhanced by KCN (a Cyt pathway inhibitor) but reduced by SHAM (an AOX inhibitor). However, TMV-induced CN-resistant respiration was increased by 18.7% in KCN+cPTIO+TMV treatment but decreased by 42.3% in DEA/NO+SHAM+TMV treatment over that of the unpretreated plant. Interestingly, the *AOX1b* transcript level was significantly increased for both treatments.

Because TMV-induced AOX induction is enhanced by NO but blocked by NO elimination, we determined whether TMV-induced NO synthesis is affected by mitochondrial electron transport (Fig. 4). The confocal laser scanning microscope (CLSM) method was used to detect the changes in NO in leaves. There was an increase in green fluorescence signal in TMV-inoculated plants when compared with that of mock-inoculated plants (Fig. 4A and B). Because the green fluorescence detected with CLSM disappeared after addition of 100 μ M cPTIO, the visual signal could be ascribed to NO accumulation. Pretreatments with either KCN or SHAM had no significant effect on TMV-induced NO production (Fig. 4C and D), suggesting that mitochondrial electron transport was not required for NO synthesis. Application of NO donor in either DEA/NO+TMV or DEA/NO+SHAM+TMV treatments resulted in a significant increase of fluorescence over TMV treatment, and the increases were substantially reduced by the pretreatment with the NO scavenging compounds (Fig. 4E to H). Similar effects of KCN and SHAM on leaf NO accumulation were observed when an independent Greiss reagent method was used for detection of NO (Fig. 4I).

Taken together, TMV-induced AOX induction in uninoculated tomato leaves is NO-sensitive while TMV-induced NO evolution is not affected by KCN or SHAM. These results

Fig. 2. Changes in leaf nitric oxide (NO) content in upper uninoculated leaves of tomato plants postinoculation with *Tobacco mosaic virus* (TMV) on a lower leaf. NO content was assayed with Greiss reagent at the time indicated. Open symbols indicate mock plants and closed symbols indicate TMV-inoculated plants. Results are mean values $\pm$ standard deviation; $n = 5$.

suggest that NO is required for systemic induction of AOX by TMV.

Levels of TMV susceptibility negatively correlate with AOX capacity in uninoculated tomato leaves.

We next investigated whether NO-regulated AOX induction is part of TMV-induced defense response in tomato plants. Because TMV infection causes a reduction in operating efficiency of photosystem II (PSII), chlorophyll fluorescence imaging method was used to analyze the response of photochemical quantum yield at PSII (Φ_{PSII}) to AOX and NO chemical modulators in TMV-infected plants. At 10 dpi, pretreatment with KCN, DEA/NO, or KCN+cPTIO all greatly increased Φ_{PSII} values when compared with unpretreated plants (Fig. 5 B, C, E, and G). In addition, the central leaves of pretreated plants did not show any symptoms of systemic infection. By contrast, pretreatment with SHAM or cPTIO substantially decreased Φ_{PSII} and caused

Fig. 3. A, Cyanide (CN)-resistant respiration and transcript levels of B, alternative oxidase (*AOX1a*); C, *AOX1b*; and D, *AOX1c* in upper uninoculated leaf of tomato plants. The upper uninoculated leaves were subjected to nitric oxide or AOX chemical modulator application for four successive days before *Tobacco mosaic virus* (TMV) inoculation. Leaf sample was collected at 4 days postinoculation. Results are mean values $\pm$ standard deviation; $n = 4$. Different letters depict significant differences between the treatments ($P < 0.05$).

enhanced leaf necrotic lesions, crinkle, and distortion (Fig. 5B, D, F, and H). Even though uninoculated leaves pretreated with DEA/NO+SHAM had higher Φ_{PSII} than unpretreated plants, TMV challenge did lead to visible symptoms of systemic infection such as necrotic lesions, mosaics, and vein chlorosis (not shown). Furthermore, real-time quantitative RT-PCR was used to determine the TMV RNA accumulation after different treatments (Fig. 5I). Generally speaking, the results of transcript levels of *TMV-coat protein (CP)* were in good agreement with those of chlorophyll fluorescence imaging analysis. Pretreatment with KCN and DEA/NO resulted in an approximately fourfold reduction of *TMV-CP* mRNA accumulation, whereas SHAM and cPTIO increased *TMV-CP* transcripts by 264.2 and 65.1%, respectively, compared with that in the unpretreated plants. While at the early stage of inoculation (at 4 dpi), *TMV-CP* transcripts in the uninoculated leaves were very low and were not significantly different among the treatments (Fig. 5I). Thus, in upper uninoculated tomato leaves, NO contents and AOX capacity were associated with TMV susceptibility (Figs. 3 to 5), and CN-resistant respiration was generally negatively correlated to the extent of TMV susceptibility.

Effects of TMV inoculation and NO on mitochondrial aconitase activity, citrate content, and COX activities in uninoculated leaves.

To investigate the role of NO in TMV-induced AOX induction, we examined the mitochondrial aconitase (ACO) activity, citrate content of TCA cycle, and COX activities of the mitochondrial electron transport chain (Fig. 6). TMV infection resulted in inhibition of ACO activity by 38.0%, which was increased to 55.7% if the TMV-inoculated plants were treated with DEA/NO but alleviated if treated with cPTIO. For mitochondrial citrate contents, a contrasting change was observed; TMV infection caused an increase in citrate content by 33.9%, which was increased to 82.8% if pretreated with DEA/NO. There was no significant difference in ACO activity and mitochondrial citrate contents between mock inoculation and cPTIO+TMV treatment.

Similar to the change of ACO activity, TMV treatment led to a decrease in COX activity by 51.4%, which was further decreased to 76.3% with DEA/NO pretreatment. However, this effect was completely abolished by the NO scavenger, and the COX activity in cPTIO pretreated plants was 91.5% higher

Fig. 4. Effects of different chemical pretreatments and *Tobacco mosaic virus* (TMV) infection on leaf nitric oxide (NO) contents in upper uninoculated leaves of tomato plants. The upper uninoculated leaves were subjected to NO or alternative oxidase (AOX) chemical modulator application for four successive days before TMV inoculation. The NO assay was made at 4 days postinoculation. **A to H,** Representative NO visualization detected using diamine fluorescein diacetate (DAF-2DA) and a confocal microscope in which NO exhibits green fluorescence. The chemical pretreatment is shown above each image: **A,** Mock; **B,** TMV; **C,** 1 mM potassium cyanide (KCN)+TMV; **D,** 3 mM salicylhydroxamic acid (SHAM)+TMV; **E,** 1 mM diethylamine NONOate (DEA/NO)+TMV; **F,** 0.2 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)+TMV; **G,** 1 mM KCN+0.2 mM cPTIO+TMV; **H,** 1 mM DEA/NO +3 mM SHAM+TMV. All images are at the same magnification and the white scale bar indicates 25 μm. **I,** NO contents assayed with Greiss reagent. Results are mean values $\pm$ standard deviation; $n = 4$. Different letters depict significant differences between the treatments ($P < 0.05$).

than untreated plants and was comparable with that of mock-inoculated plants.

DISCUSSION

Increase in AOX gene expression or NO production is frequently observed in both compatible and incompatible plant-pathogen interactions (Lennon et al. 1997; Chivasa and Carr

1998; Floryszak-Wieczorek et al. 2007; Love et al. 2007; Király et al. 2008). We showed that CN-resistant respiration and NO are increased within 12 h in upper uninoculated leaves of susceptible tomato plants after inoculation with TMV. Furthermore, TMV-induced AOX induction is enhanced by NO and blocked by NO scavenger. However, this TMV-induced NO production did not require AOX activation. Exogenous KCN- or NO-induced CN-resistant alternative respiration is

Fig. 5. Effects of different chemical pretreatments and *Tobacco mosaic virus* (TMV) infection on leaf photochemical quantum yield at photosystem II (PSII) (Φ_{PSII}) and *TMV-coat protein* (CP) gene transcription in upper uninoculated leaves of tomato plants. The upper uninoculated leaves were subjected to nitric oxide or alternative oxidase (AOX) chemical modulators application for four successive days before TMV inoculation. The Φ_{PSII} image was made at 10 days postinoculation whereas the *TMV-CP* transcripts assay was made at both 4 and 10 days postinoculation. The chemical pretreatment is shown above each image. **A to H,** Representative Φ_{PSII} images of from the IMAGING-PAM. **A,** Mock; **B,** TMV; **C,** 1 mM potassium cyanide (KCN)+TMV; **D,** 3 mM salicyl-hydroxamic acid (SHAM)+TMV; **E,** 1 mM diethylamine NONOate (DEA/NO)+TMV; **F,** 0.2 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)+TMV; **G,** 1 mM KCN +0.2 mM cPTIO +TMV; **H,** 1 mM DEA/NO +3 mM SHAM+TMV. The circle in (A) to (H) indicates the spot where the fluorescence and aperture measurements were performed shown as the data in each figure. Scale bar = 0.5 cm. The color gradient scale below indicates the magnitude of the fluorescence signal represented by each color. **I,** Transcript levels of *TMV-CP* gene by real-time reverse-transcription polymerase chain reaction. Results are mean values $\pm$ standard deviation; $n = 4$. Different letters depict significant differences between the treatments ($P < 0.05$).

generally negatively correlated to levels of TMV susceptibility. Therefore, our results establish a connection between AOX and NO in regulation of a systemic antiviral defense response in tomato and show that NO acts upstream and mediates induction of AOX, which then contributes to limitation of virus systemic infection and accumulation.

The role of AOX and NO in systemic basal defense to TMV in tomato plants.

In this study, application of KCN and DEA/NO at nonlethal concentrations to uninoculated leaves can induce resistance to TMV and the resistance was totally antagonized by SHAM or cPTIO (Fig. 5). Increases in NO synthase (NOS)-like activity and the role of NO in systemic acquired resistance have been studied previously in tobacco plants challenged with TMV (Durner et al. 1998; Klessig et al. 2000; Song and Goodman 2001) and in *Arabidopsis* plants inoculated with *Pseudomonas syringae* (Feechan et al. 2005; Tada et al. 2008). Our results

Fig. 6. Effects of *Tobacco mosaic virus* (TMV) infection and pretreatments with diethylamine NONOate (DEA/NO) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) on **A**, mitochondrial aconitase activity; **B**, citrate content; and **C**, cytochrome *c* oxidase (COX) activity in upper uninoculated leaves of tomato plants. The upper uninoculated leaves were subjected to DEA/NO and cPTIO application for four successive days before TMV inoculation. Leaf sample was collected at 4 days postinoculation. Results are mean values $\pm$ standard deviation; $n = 4$. Different letters depict significant differences between the treatments ($P < 0.05$).

further support the hypothesis that NO plays an important role in plant defense responses. However, there has been no conclusive evidence for CN-resistant respiration in systemic basal defense against viral pathogens. In this work, we found that systemic TMV infection could be diminished by KCN and enhanced by the AOX inhibitor and that KCN could fully alleviate cPTIO-induced Φ_{PSII} decrease and TMV-CP accumulation (Fig. 5). Most importantly, both AOX and CN-resistant respiration appeared to be negatively correlated to the magnitude of TMV susceptibility (Figs. 3 and 5). In accordance with this result, SHAM treatment accelerated compatible *Cauliflower mosaic virus* (CaMV) spread in *Arabidopsis* and partially restored susceptibility of several mutants to wild-type levels (Love et al. 2007). Our results, together with previous observations, suggest that AOX induction contribute positively to systemic basal defense against viral pathogens in plants (Chivas and Carr 1998; Naylor et al. 1998; Singh et al. 2004).

As in environmental stresses such as chilling, ozone, or treatment with exogenous elicitors such as harpin, the potential means by which AOX acts on virus attack is by modulating mitochondrial (or cellular) redox and reducing ROS production (Murphy et al. 1999; Ederli et al. 2006; Noctor et al. 2006; Vidal et al. 2007; Watanabe et al. 2008). AOX lowers buildup of ROS in the mitochondria by preventing over-reduction of the cytochrome respiratory electron transport chain and the ubiquinone pool (Maxwell et al. 1999; Navrot et al. 2007). The AOX-mediated changes in ROS production or cellular redox status may regulate the activity of downstream elements in the SHAM-sensitive pathway. This model is based on the observation that the *Nicotiana sylvestris* mitochondria mutants, which have elevated levels of AOX protein, have an altered redox status, and expression of antioxidant enzymes showed enhanced TMV resistance with reduced number and size of lesions (Dutilleul et al. 2003). However, overproduction of wild-type or mutant AOX protein accelerated systemic movement of TMV in *N. benthamiana* (Murphy et al. 2004). These results indicate that abnormal levels of AOX proteins could perturb the mitochondrial redox balance, which then compromises plant defense against virus (Király et al. 2008). In addition, AOX induction did not induce resistance to CMV in squash (Mayers et al. 2005). In *Arabidopsis*, if antimycin A treatment and viral inoculation were on separate leaves, the plant susceptibility to compatible CaMV was not reduced, even though inoculation of virus and treatment of antimycin A on the same leaf effectively induced *Arabidopsis* resistance to CaMV (Love et al. 2005). Therefore, discrepancy between these reports and our results suggest that different host species might use different approaches in defense against different viral pathogens.

Interrelationship between systemic AOX induction and NO generation in response to TMV inoculation.

In this study, we observed that treatment with DEA/NO and cPTIO resulted in AOX induction and suppression, respectively, and that the levels of virus susceptibility could be antagonized by SHAM and KCN (Figs. 3 and 5). These observations suggest a possible connection between AOX and NO in modulating defense response. Previously, it was found that *AOX1a* expression and CN-resistant respiration were strongly induced in *Arabidopsis* cell cultures after treatment with NO (Huang et al. 2002). Similarly, Ederli and associates (2006) also demonstrated that, in O_3 -fumigated tobacco plants, NO-dependent pathway was required for O_3 -induced upregulation of *AOX1a*. However, mitochondrial electron transport and AOX pathway were identified as major sources of NO synthesis because strong inhibition of NO generation was achieved by SHAM in *N. tabacum* and unicellular green alga *Chlorella*

sorokiniana (Tischner et al. 2004; Planchet et al. 2005). These results suggest that there is a cross-talk between NO and AOX in plant cells in response to stresses or stimuli. In the present study, our data suggest that NO acts upstream of TMV-induced AOX induction. First, time-course analysis of NO generation and AOX induction showed that the increase in NO generation preceded AOX induction in response to TMV infection (Figs. 1 and 2). Second, DEA/NO significantly increased transcript levels of AOX genes, and pretreatment with cPTIO almost fully blocked TMV-induced upregulation of AOX gene expression and CN-resistant respiration (Fig. 3). By contrast, pretreatments with an AOX inhibitor had little effect on NO production (Fig. 4). Therefore, it is most likely that TMV-induced NO triggers AOX induction, and this induced AOX then plays a role in regulating systemic basal defense response against TMV.

The mobile signals involved in the systemic defensive system is unclear but may not be the virus itself because, at 4 dpi the *TMV-CP* mRNA accumulation in the uninoculated leaf tissue did not differ between treatments (Fig. 5I), which is consistent with previously reported leaf removal experiments (Love et al. 2005). Furthermore, tobacco epidermal cells respond to both incompatible and compatible isolates of TMV within seconds of exposure (Allan et al. 2001). An ROS burst occurs both locally and systemically within 2 to 6 h of inoculation with compatible CaMV to *Arabidopsis* (Love et al. 2005). In the present study, TMV inoculation to the lower leaves induced a rapid NO synthesis and AOX activation in upper uninoculated leaves as early as 0.5 dpi (Figs. 1 and 2). Therefore, in addition to NO and AOX, ROS might be involved in the systemic defensive system.

It should be noted that the DEA/NO-induced TMV resistance was not fully abolished by SHAM (Fig. 5); thus, an additional unknown AOX-independent pathway in the NO signaling pathway might exist. In addition, several lines of evidence pointed out that SA, ethylene (ET), and other signaling molecules may also be involved in AOX induction (Lennon et al. 1997; Norman et al. 2004; Ederli et al. 2006). However, NO-induced AOX was not affected after treatment with NO in the *pad4* and *npr1* mutants and in the transgenic *NahG* plants (Huang et al. 2002); antimycin A-induced resistance to CaMV was not affected in *NahG* plant and ET-signaling mutant *etr1* (Love et al. 2007). Therefore, it is likely that SA, NO, and ET act synergistically or independently in AOX induction. Accordingly, further experiments are required to fully establish the relationship between SA, NO, ET, and their possible functions in AOX induction and TMV resistance.

Potential mechanism of NO on AOX induction under TMV-inoculated conditions.

How the TMV-induced NO regulates induction of AOX and the alternative pathway respiratory O₂ uptake is unclear. NO-mediated expression of defense genes in plants is through both cyclic guanosine monophosphate (cGMP)-dependent and cGMP-independent pathways, the latter involving the activation of thiol-containing proteins and Tyr nitration, or through the direct interaction of NO with metal-containing proteins (Kroncke et al. 1997). Thus, NO-induced AOX in mitochondria may involve a mechanism by which NO targets the iron-sulfur cluster-dependent enzymes ACO of the TCA cycles, or the heme-containing enzyme COX, the Cyt pathway terminal member of the mitochondrial electron transport (Millar and Day 1996; Navarre et al. 2000). In our study, both mitochondrial ACO and COX activity was inhibited by TMV and this inhibition was significantly enhanced by DEA/NO and eliminated by cPTIO (Fig. 6). ACO catalyzes the reversible isomerization of citrate to isocitrate; thus, inactivation of ACO not

only affects mitochondrial energy metabolism but also results in elevated citrate concentrations (Shlizerman et al. 2007). Interestingly, exogenous application of citrate to tobacco suspension cells caused a rapid and dramatic increase in the steady-state level of *Aox1* mRNA and AOX protein (Vanlerberghe and McIntosh 1996). The elevated citrate content might play a role in TMV-induced AOX induction in the present study. In addition, COX has been reported as another primary target of NO because endogenous NO inhibited cytochrome *c*-dependent respiration but not the AOX in isolated soybean mitochondria (Millar and Day 1996; Yamasaki et al. 2001). The alternative respiratory pathway was subsequently induced to maintain energy metabolism and ubiquinone pool at low steady-state poise when electron flow through the Cyt pathway is restricted (Millar and Day 1996, 1997). Because a direct interaction between NO and AOX appear unlikely, it is possible that AOX may act as a cellular component to receive the NO signal indirectly. This hypothesis is supported by previous observations that activation of the NO-tolerant alternative respiratory pathway could dampen the generation of ROS and maintain mitochondrial (or cellular) function when NO-sensitive ACO and COX were inhibited (Millar and Day 1997; Maxwell et al. 1999). This might be a role of AOX during plant-virus interactions. It should be mentioned that antioxidant induction has also been suggested to play important roles in virus-induced plant defense reactions (Király et al. 2002; Després et al. 2003). Therefore, reduction of ROS accumulation during virus attacks might be achieved by both AOX (elimination of ROS production) and antioxidants (elimination of ROS production).

In summary, we demonstrated in this study that mitochondrial AOX and NO crosstalk in induction of antiviral defense response in tomato uninoculated leaves against TMV infection. The results suggest that TMV-induced NO generation acts upstream and mediates AOX induction, which might then modulate mitochondrial (or cellular) redox and reduce ROS production, thus limiting virus systemic infection and accumulation.

MATERIALS AND METHODS

Plants, virus, and chemical treatments.

Tomato (*Solanum lycopersicum* L. cv. Hezuo 903, TMV susceptible) plants were grown in a greenhouse at 25 and 20°C (day and night air temperature, respectively) with a 16-h photoperiod. Three upper fully developed leaves of four- to five-leaf-stage seedlings were used for chemical pretreatment, and the lower fully developed leaves were used for inoculation with TMV 4 days after chemical pretreatment. Stock solutions of cPTIO and KCN were prepared fresh weekly in 20 mM potassium phosphate buffer, pH 6.8, and SHAM was dissolved in deionized water. A stock solution of DEA/NO was made in 10 mM NaOH on ice and prepared daily. To initiate the release of NO, an aliquot of the stock alkaline solution was dissolved in 10 mM phosphate buffer, pH 7.4, to give a 1-mM final concentration. Tomato plants were sprayed with water or freshly diluted solutions of 1 mM KCN, 3 mM SHAM, 1 mM DEA/NO, 0.2 mM cPTIO, 1 mM KCN plus 0.2 mM cPTIO, or 1 mM DEA/NO plus 3 mM SHAM daily in both the adaxial and abaxial surface. Four days later, the lower fully developed leaf of each plant was inoculated with the U1 strain of TMV. Viral inoculum was prepared in 5 mM potassium phosphate buffer, pH 7.2, at 6 µg ml⁻¹. For inoculation, the plants were sprayed with Carborundum and then inoculated by rubbing 100 µl of viral inoculum onto each leaf with cotton swabs. Mock-inoculation controls were prepared using the same procedure with buffer. The upper newly developed uninoculated leaves were collected at different time points as indicated for

leaf respiration and internal NO content assays. Unless otherwise stated, the physiological and molecular measurements were carried out 4 dpi and the chlorophyll fluorescence imaging was made at 10 dpi, while *TMV-CP* gene transcription analysis were made both at 4 and 10 dpi. Experiments were independently carried out three times.

Respiration in uninoculated leaves.

Respirations of leaves were measured following the method of Millenaar and associates (2002) using a Clark-type oxygen electrode (Hansatech, King's Lyn, U.K.) at different days postinoculation. Plants were kept in darkness for 15 min before respiration measurement and leaves were detached and cut into pieces with a razor blade. Leaf samples (0.1 g) were transferred to air-tight cuvettes containing 2 ml of air-saturated 20 mM potassium phosphate buffer (pH 6.8) and respiration was measured as a decrease of O₂ concentration at 25°C. CN-resistant respiration was measured in the presence of 1 mM KCN.

RNA extraction and transcript level estimation by real-time quantitative PCR.

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's specifications. Genomic DNA was removed using the RNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using 2 µg of RNA with the RevertAid first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Gene-specific primers were designed for analyzing transcript levels of each member of the AOX family. *TMV*-specific primers were also designed based on the sequence encoding the *TMV-CP*. The primers used for the amplification of each cDNA are listed in Table 1.

RT-PCR was performed with an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.). Each reaction (20-µl total volume) consisted of 10 µl of iQ SYBR Green Supermix, 1 µl of diluted cDNA, and 0.1 µM each of forward and reverse primers. PCR cycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 58°C for 45 s. Fluorescence data were collected during the 58°C step. The tomato *actin* gene was used as an internal control. Relative gene expression was calculated as described by Livak and Schmittgen (2001).

Measuring NO accumulation.

NO detection by a CLSM system was carried out essentially following the method of Corpas and associates (2004) with minor modifications. Leaf sections (0.5 by 0.5 cm) were taken using a razor blade from the studied leaves and incubated in the dark for 30 min at 25°C in 10 mM Tris-HCl (pH 7.4) containing 10 µM 4,5-diaminofluorescein diacetate (DAF-2DA) (Sigma-Aldrich, St. Louis). After washing twice in fresh buffer for 15 min each, the leaf sections were

mounted in the same buffer on microscope slides and then examined immediately under a CLSM system (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany). The sections were excited with the 488-nm line of an argon laser and dye emissions were recorded using a 505- to 530-nm band-pass filter. Background staining was controlled with leaf sections unstained or preincubated with 1 mM cPTIO (1 h, 25°C), a NO scavenger. In addition, NO was also determined with Greiss reagent (Sigma-Aldrich) following the method of Zhou and associates (2005).

Chlorophyll fluorescence imaging.

Leaves used for chlorophyll fluorescence assay were collected 10 dpi so that infection of *TMV* could be analyzed with the MAXI version of the IMAGING-PAM M-Series chlorophyll fluorescence system (Heinz-Walz GmbH, Effeltrich, Germany). This system allows noninvasive determination of the quantum efficiency of PSII by the saturation pulse method (Genty et al. 1989). Saturation pulses were given every 20 s and Φ_{PSII} was calculated as $(F_m' - F_t)/F_m'$ (Genty et al. 1989).

Enzyme activities and citric acid content assays.

ACO activity was determined with mitochondrial fractions following the protocol for mitochondria isolation by Mittova and associates (2000) with some modifications as described previously (Shi et al. 2008). After mitochondria were isolated and broken up, ACO was purified according to Navarre and associates (2000) and was measured spectrophotometrically at 240 nm by monitoring formation of *cis*-aconitate from isocitrate (Kennedy et al. 1983). The assay was performed at room temperature in 75 mM Tris-Cl (pH 8.0) containing 30 mM DL-trisodium isocitrate. An extinction coefficient for *cis*-aconitate of 3.6 mM⁻¹ cm⁻¹ at 240 nm was used. COX activity (EC 1.9.3.1) was measured at 550 nm (25°C) in the presence of 20 µM reduced cytochrome *c* in 10 mM phosphate buffer (pH 7.2) according to Millenaar and associates (2002).

Citric acid content in leaf tissue was analyzed by high performance liquid chromatography (HPLC) as described by Suárez and associates (2008). Samples (10 µl) were loaded onto the Waters HPLC System (Waters Corporation, Milford, MA, U.S.A.) with a spherisorb C18 column (4.6 by 250 mm). Temperature of the column was set at $T = 35^\circ\text{C}$ during all of the experiments. The mobile phase was 20 mM KH₂PO₄, adjusted pH to 2.5 with concentrated H₃PO₄ at a flow rate of 1 ml min⁻¹. Citric acid peaks were detected with a PDA detector at 210 nm.

Statistical methods.

There were at least three independent replicates for each determination. Data were subjected to analysis of variance and the means were compared using Tukey's test at 5% level.

Table 1. Primers used for real time reverse-transcription polymerase chain reaction assays

Gene	Encoding protein	Accession no.	Primer pairs
<i>TMV-CP</i>	<i>Tobacco mosaic virus</i> coat protein	V01408.1	F: 5'-TTCTTGTCATCAGCGTGGGC-3' R: 5'-TTCGGCAGTCGTGGGGTTC-3'
<i>AOX1a</i>	Alternative oxidase 1a	AY034148.1	F: 5'-TCATTACCAAGGACAACAGC-3' R: 5'-GGAACAAAATAGTGACGGAC-3'
<i>AOX1b</i>	Alternative oxidase 1b	AY034149.1	F: 5'-TCCTCCACTGTAAATCCC-3' R: 5'-AAATACGCCTTGAAGTGC-3'
<i>AOX1c</i>	Alternative oxidase 1c	AY324397.1	F: 5'-TAAGGCGATTTCGAGCACA-3' R: 5'-CCATTAAGACAAGTCCACGT-3'
<i>AOX2</i>	Alternative oxidase 2	AY324396.1	F: 5'-ATGATATTGATCGTGTTGA-3' R: 5'-TCCTTAGGCAGTCTCCA-3'
<i>Actin</i>	...	U60481.1	F: 5'-TGTCCCTATTACGAGGGTATGC-3' R: 5'-CAGTTAAATCACGACCAGCAAGAT-3'

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