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Alternative oxidase in resistance to biotic stresses: *Nicotiana attenuata* AOX contributes to resistance to a pathogen and a piercing-sucking insect but not *Manduca sexta* larvae

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Abstract

The role of the alternative respiratory pathway in the protection of plants against biotic stress was examined in transgenic tobacco (*Nicotiana attenuata*) plants (irAOX) silenced in the expression of *ALTERNATIVE OXIDASE (AOX)* gene. Wild-type (WT) and irAOX plants were independently challenged with: (i) chewing herbivores (*Manduca sexta*), (ii) piercing-sucking insects (*Empoasca* spp.) and (iii) bacterial pathogens (*Pseudomonas syringae* pv. tomato DC3000), showing that all these treatments can strongly elicit accumulation of *AOX* gene transcripts in WT plants. When *N. attenuata* chemical defenses and resistance were examined, irAOX plants showed WT levels of defense-related phytohormones, secondary metabolites and resistance to *M. sexta*. In contrast, piercing-sucking leafhoppers (*Empoasca* spp.) caused more leaf damage and induced significantly higher salicylic acid (SA) levels in irAOX compared to WT plants in the field and/or glasshouse. Subsequently, irAOX plants accumulated lower levels of defense metabolites, 17-hydroxygeranyllinalool diterpene glycosides, caffeoylputrescine and nicotine compared to WT plants under prolonged attack of leafhoppers in the glasshouse. Finally, an accelerated cell death phenotype was observed in irAOX plants infected with *P. syringae*, which correlated with higher levels of SA and hydrogen peroxide levels in pathogen-infected irAOX compared to WT leaves. Overall, the AOX-associated changes in phytohormone and/or redox levels appear to support the resistance of *N. attenuata* plants against cell piercing-sucking insects and modulate the progression of cell death in pathogen-infected tissues but are not effective against rapidly feeding specialist herbivore *M. sexta*. 
Introduction

Metabolic plasticity allows plants to adapt to variable environmental stress conditions. Plants rapidly reconfigure their primary metabolism during stress to cope with the increased metabolic demands of resistance responses, and mitochondria are known to play a central role in this reconfiguration (reviewed in Bolton, 2009; Millar et al., 2011). Under stress, electrons are frequently re-routed through an alternative respiratory pathway branching from the cytochrome (Cyt) pathway at the level of ubiquinone (UQ) pool to an enzyme known as the alternative oxidase (AOX; McDonald, 2008). In contrast to the Cyt pathway, the AOX pathway is resistant to cyanide and therefore known as cyanide-resistant respiration (Henry and Nyns, 1975).

The alternative pathway bypasses two important energy conservation sites (complex III and IV) and avoids oxidative phosphorylation and adenosine triphosphate (ATP) synthesis: energy is dissipated as heat after reduction of oxygen to water by AOX enzyme (Moore et al., 1978). While alternative respiration is widespread in plants, fungi and some prokaryotes (McDonald, 2008), its physiological function and seemingly energy-wasting character are not well understood.

One of the most investigated functions of AOX is heat production which, for example, can volatilize odoriferous compounds during flowering for the attraction of pollinators, for example in Arum lily flowers (Meeuse, 1975; Raskin et al., 1987). Heat production is only associated with a limited number of thermogenic plant species (Seymour, 1997), and alternative functions of AOX have been extensively investigated. Subsequently, AOX has been established as one of the essential defense components in plant response to acute stress (Arnholdt-Schmitt et al., 2006). Abiotic stresses such as drought (Bartoli et al., 2005), high salt (Costa et al., 2007; Feng et al., 2010a), low temperature (Vanlerbergh and McIntosh, 1992; Popov et al., 2011; Wang et al., 2011) and wounding (Hiser and McIntosh, 1990) are known to stimulate the activity of alternative respiratory pathway or at least increase AOX transcripts and/or protein levels. Phytochrome, phototropin and cryptochrome photoreceptors have been shown to mediate the light-responsiveness of the AOX1a gene in Arabidopsis.
seedlings and aox1a mutants showed increased photobleaching damage under high light conditions (Zhang et al., 2010). Several studies have indicated that AOX may also function in resistance to biotic stress such as infection by pathogens. For example, AOX protein levels increased in local and systemic tobacco, and in tomato leaves infected with tobacco mosaic virus (TMV) (Lennon et al., 1997; Liao et al., 2012). The overexpression of AOX protein resulted in smaller HR lesions, suggesting that AOX may act as a suppressor of programmed cell death (PCD) in virus-infected leaves (Ordog et al., 2002). The induction of AOX in mustard during infection with Turnip mosaic virus was suppressed in the presence of ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG), suggesting that ethylene and AOX are likely involved in leafy mustard’s systemic resistance to virus infection (Zhu et al., 2011).

Bacterial pathogens and their elicitors also strongly elicit AOX transcript accumulations and protein levels, both in leaves and cell suspensions (Krause and Durner, 2004; Mizuno et al., 2005; Kiba et al., 2008). In Arabidopsis plants, infection with virulent and avirulent strains of Pseudomonas syringae pv. tomato elevated AOX transcript and proteins levels in infected leaves (Simons et al., 1999). An accurate oxygen isotope discrimination method was used to determine the partitioning of electrons between Cyt and the alternative pathways in pathogen-infected plants and documented a 5-fold increase in in vivo AOX activity 4 h after application of the bacterial elicitor harpin N$_{Ea}$ to Nicotiana sylvestris leaves (Vidal et al., 2007). In Paracoccidioides brasiliensis, a human thermal dimorphic pathogenic fungus, PbAOX played important role in fungal defense response against oxidative stress imposed by immune cells and virulence of the pathogen (Ruiz et al., 2011).

Control of oxidative damage is therefore essential for living organisms including plants to survive under/and recover from stresses (Feng et al., 2010b). Here, AOX is very likely to counteract the over-reduction of mitochondrial UQ pools by consuming excess reducing equivalents, particularly when the flow of electrons through the Cyt pathway is impaired by acute stress. Because insects and microbial attack are both knowingly associated with oxidative bursts (Torres et al. 2002; De Vos et al., 2007; Maffei et al., 2007), AOX and its redox-controlling function could contribute to a
plant’s resistance to herbivores and pathogens. To test this expectation, we transformed *Nicotiana attenuata* plants with an RNAi construct harboring a fragment of *NaAOX* gene in an inverted repeat orientation (irAOX) to silence the expression of endogenous *NaAOX* genes and selected three mechanistically distinct biotic stress models to thoroughly examine the role of AOX in plant defense against biotic stresses. While *NaAOX* transcript and protein levels significantly increased in response to all three stress conditions, AOX played different roles in response of plants to these different biotic stresses.

**Results**

*Identification of AOX gene sequences in N. attenuata*

Two AOX genes, *NaAOX1* and *NaAOX2* from *N. attenuata* of nearly identical nucleotide sequence have been previously deposited in public NCBI databases (AY422688; AY422689). Both genes show a high level of identity to members of the *A. thaliana* AOX1 gene family: NaAOX1 and NaAOX2 proteins are 70.7 and 70.3 % identical to AtAOX1a from Arabidopsis (Supplemental Fig. S1A). The high degree of identity between *NaAOX1* and *NaAOX2* genes at the nucleotide level did not allow for efficient monitoring and gene-specific silencing of individual NaAOX isoforms by reverse transcription (RT) quantitative PCR (RT-qPCR) and RNA interference, respectively. Therefore, primers detecting transcripts of both genes were used and reported herein as *NaAOX* transcript abundance. Subsequently, both genes were targeted with a single silencing construct to down regulate AOX expression. Despite our attempts to identify additional AOX genes from *N. attenuata*, we were not able to find any additional conserved AOX proteins in this diploid tobacco species.

*Analysis of NaAOX transcript levels in response to attack from a chewing herbivore*

Abiotic stress and pathogen elicitors are known to increase transcript levels of AOX genes in plants. However, much less is known about the effects of herbivory. We therefore examined *NaAOX* transcript levels in WT leaves subjected to wounding,
simulated herbivory treatment, and after direct feeding of the *N. attenuata* leaves by *M. sexta* neonates. The NaAOX transcripts strongly increased 1h after wounding of *N. attenuata* leaves with a pattern wheel and immediately applying either water to mechanical wounds (W+W) or *M. sexta*’s oral secretions (OS) to simulate herbivory (W+OS) (Figure 1A; Supplemental Figure S1B). Interestingly, NaAOX transcript and protein levels appeared higher in W+OS- compared to W+W-treated leaves at 1-3 h (Figure 1A ; Supplemental Figure S2A). Similarly, direct *M. sexta* feeding significantly elevated basal NaAOX transcript and protein levels at 1 and 2 d after placing neonates on the leaves (Fig. 1B; Supplemental Figure S2B). Because NaAOX transcript levels increased significantly more in response to *M. sexta*’s salivary elicitors, we hypothesized that AOX may function in direct defense of *N. attenuata* against chewing herbivores. In addition to the herbivore- and wound-regulated patterns shown in Fig. 1, an apparent oscillation in NaAOX transcript abundance was observed in untreated control leaves, suggesting a possible circadian control of AOX transcription (Fig. 1A). In particular, AOX transcripts appeared to be higher during the light periods than during dark periods, which could be associated with a previously reported AOX function in buffering high photosynthetic and respiration rates in response to high light (Dinakar et al., 2010).

**NaAOX silencing does not compromise defense of *N. attenuata* against *M. sexta* caterpillars**

To examine the function of NaAOX in resistance of native tobacco against its specialist herbivore *M. sexta* caterpillars, we generated stably AOX-silenced *N. attenuata* plants (irAOX) using RNA interference and inverted repeat fragment of the *NaAOX1* gene (Supplemental Fig. S3A). Two independent homozygous diploid lines (referred to as irAOX-200 and irAOX-203) carrying a single insertion of T-DNA were selected for further experiments (Supplemental Fig. S3B). In both lines, the levels of NaAOX transcripts were strongly reduced compared to identically treated WT plants (Supplemental Fig. S3C). Alternative respiration capacity of the leaves was also significantly suppressed in irAOX plants compared to WT plants (Supplemental Fig.
Feeding of *M. sexta* larvae elicits burst of JA and JA-Ile in *N. attenuata* leaves, which triggers defense signaling pathways and deploys defense responses (Wu and Baldwin, 2010). To examine if NaAOX mediates changes in phytohormone levels, we determined JA and JA-Ile contents in irAOX and WT plants at 0, 45, 90, and 180 min after W+W- and W+OS-elicitation. Similar analysis was conducted using *M. sexta*-attacked leaves before and 1, 2, and 3 d after placing neonates on the leaves. While both treatments elicited significantly higher levels of JA and JA-Ile in the leaves compared to untreated controls, we did not find any significant differences in JA or JA-Ile content between WT and irAOX plants (Fig. 2A). The levels of another stress hormone, salicylic acid (SA) remained comparable between WT and irAOX plants (Supplemental Fig. S5A, B) and staining of W+OS and control leaves with DAB showed comparable levels of hydrogen peroxide accumulation in WT and irAOX leaves (Supplemental Fig. S5C). Because important defense metabolites such as nicotine (Steppuhn et al., 2004) and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs; Jassbi et al., 2008; Heiling et al., 2010) were not differentially regulated in irAOX compared to WT plants (Supplemental Fig. S6), it was not surprising that caterpillar performance in feeding bioassays did not differ (Fig. 2B). These results suggest that despite increased transcript levels of NaAOX in response to *M. sexta* attack, NaAOX genes do not play any significant role in the direct defense of *N. attenuata* plants against chewing specialist herbivores. The induction of NaAOX transcripts and protein levels by insect feeding could be a consequence of defense against mechanical wounding, similar to previously described accumulation of AOX protein in mechanically wounded potato (*Solanum tuberosum*) tubers (Hiser and McIntosh, 1990).

We also considered an alternative hypothesis that increased AOX expression during hervivory could facilitate higher emissions of volatile organic compounds (VOCs), mediators of indirect defenses, from herbivore-attacked leaf surfaces of *N. attenuata*. However, in our previous experiments we did not find any consistent differences in α-bergamotene or benzyl acetone emissions from WT and irAOX
plants, even when the plants were maintained at lower than ambient temperatures (data not shown).

**NaAOX silencing makes plants more susceptible to piercing-sucking leafhoppers**

In the natural environment, plants are exposed to a large variety of pests and pathogens. When irAOX-203 plants were transplanted as size-matched pairs with *N. attenuata* WT plants into their native habitat in Southwestern Utah (Great Basin Desert, USA), we noticed that leafhoppers (*Empoasca* spp.) caused significantly more damage on irAOX-203 compared to WT plants (Fig. 3A). We also observed mirid- and flee beetle-associated damage on the plants in the field; in contrast to leafhopper damage, there were no significant differences in damage caused by these insects (data not shown).

The *Empoasca* leafhoppers are cell rupture feeders that are often associated with microbes transmitted during herbivore feeding (Backus et al. 2005). Although *Candidatus* Phytoplasma spp. have not been identified in *Empoasca* spp. occurring in the Utah field or in our in-house colony derived from Utah collected insects (Kallenbach et al., 2012), it suggests that AOX is important for protection of plants against sucking-piercing insects and/or other microbes, such as viruses and bacteria, vectored by these insects. To examine the robustness of our initial field observations, a choice-test experiment with irAOX and WT plants was conducted in the glasshouse. An equal number of WT and irAOX-203 plants were placed in a randomized design in the sealed glass container with *Empoasca* spp. leafhoppers that were allowed to feed on the plants for 12 d. Consistent with our field observations, irAOX plants were significantly more damaged compared to WT plants (Fig. 3B).

The *NaAOX* transcript and protein levels were determined before and after 4, 8, and 12 d of exposure of WT plants to *Empoasca* spp. feeding (Fig. 4; Supplemental Fig. S2C). Leafhopper feeding induced *NaAOX* transcripts that became 6-fold more abundant compared to control plants after 12 d of exposure (Fig. 4). The consistently higher damage on attacked irAOX plants (Fig. 3B) and inducible character of *NaAOX* gene shown in Fig. 4 prompted a more detailed analysis of *N. attenuata*
phytohormone and defense metabolite profiles after *Empoasca* spp. attack.

**irAOX plants contain higher levels of SA after leafhopper attack**

Piercing-sucking insects such as aphids, mites and leafhoppers can activate both salicylate- and jasmonate-dependent defense signaling pathways in plants (Ament et al., 2004; Mozoruk et al., 2006; Kempema et al., 2007). The lack of AOX activity in irAOX *N. attenuata* plants clearly disturbed the homeostasis of SA after leafhopper attack. Although SA levels did not differ in the control non-attacked WT and irAOX-203 set of plants, SA levels increased in response to *Empoasca* spp. attack in irAOX, but remained unchanged in WT plants (Fig. 5A). In contrast, JA and JA-Ile levels were not differentially elicited in WT and irAOX plants (Fig. 5B). It is likely that jasmonate levels after *Empoasca* spp. attack increased only transiently and/or locally, which was not captured in our experimental setup (first measurement after 4 d). Recently, a significant increase in jasmonate levels was reported in the leaves of *N. attenuata* challenged with 25 *Empoasca* spp. individuals allowed to feed on the leaf for 2 d (Kallenbach et al. 2012). In addition, reduced resistance to leafhoppers was found in the field and glasshouse experiments with JA biosynthesis-deficient *N. attenuata* plants (Kessler et al., 2004; Kallenbach et al., 2012). In addition, irLOX3 plants silenced in the accumulation of JA were significantly more damaged by *Empoasca* spp. compared to WT plants in our glasshouse choice test experiments (Supplemental Fig. S7), emphasizing a central role for JA in *Empoasca* spp. feeding behavior.

**Leafhopper-attacked irAOX plants have attenuated defense metabolite accumulations after prolonged feeding**

Plants use a large variety of secondary metabolites to defend against herbivores and pathogens (Baldwin 2001; Metlen et al. 2009). Nicotine, caffeoylputrescine (CP) and HGL-DTGs are typical secondary metabolites found in *N. attenuata* plants elicited with biotic stresses (Steppuhn et al., 2004; Kaur et al., 2010; Heiling et al., 2010). While nicotine and HGL-DTGs are known to strongly inhibit *M. sexta* larvae
feeding, hydroxycinnamic acid-polyamine conjugates like CP can function against both herbivores and microbes (reviewed in Bassard et al., 2010). In our experiments, nicotine (P<0.001), CP (P<0.05) and HGL-DTGs (P<0.001) accumulated significantly less in irAOX compared to WT plants exposed for 8-12 d to leafhopper feeding (Fig. 6), suggesting a positive role of AOX in efficient accumulation of these metabolites after prolonged *Empoasca* spp. feeding. The initial feeding of leafhoppers, however, is known to be independent of nicotine, HGL-DTGs and PIIs in *N. attenuata* plants (Kallenbach et al., 2012). Consistently, after 4 d of *Empoasca* spp. feeding, defense metabolites were not yet elicited (Fig. 6).

**NaAOX transcripts increase in response to pathogen infection**

The treatment of tobacco cells with pathogen-associated elicitors is known to alter partitioning between Cyt- and AOX-dependent respiratory pathways, and transiently increase AOX expression (Vidal et al., 2007). We examined if *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) strain infection can induce AOX expression in *N. attenuata*; *Pst* DC3000 causes local chlorosis followed by leaf necrosis in *N. attenuata* (Hettenhausen et al., 2012). The leaves of rosette stage plants were infiltrated with 1×10⁵ CFU suspension of *Pst* DC3000 and samples were collected from infiltrated leaf areas before and 45, 90 min, 3, 24, 48, and 72 h after pathogen inoculation. Both mock- and pathogen-inoculated leaves experienced a transient burst of NaAOX transcripts at 45 min post inoculation (Fig. 7), which was likely caused by wounding during infiltration process. However, transcript and protein levels of NaAOX dramatically up-regulated in pathogen-inoculated compared to mock-treated leaves at the later stages of infection (Fig. 7; Supplemental Fig. S2D), suggesting a direct involvement of AOX in plant defense against pathogen-imposed stress. The levels of NaAOX protein in *Pseudomonas*-infected leaves of irAOX-200 and irAOX-203 transgenic lines at 2 and 3 d remained low (Supplemental Fig. S2D) as expected from AOX-silencing efficiency shown in Supplemental Fig. S3C.

**NaAOX silencing accelerates *Pst* DC3000-induced cell death**
In the following experiment, we observed the progress of necrosis in WT and irAOX leaves inoculated with \( Pst \) DC3000. Both irAOX-200 and irAOX-203 lines exhibited moderately but reproducibly accelerated development of necrotic symptoms, which typically occurred 1 d earlier in irAOX compared to WT plants (Fig. 8A, left panel). Microscopic examination of trypan blue-stained leaves confirmed more extensive cell death in irAOX leaves 2 d after inoculation with pathogen compared to identically treated WT leaves (Fig. 8A, right panel). When the extent of cell death was estimated by quantitative electrolyte leakage of the cells (Pike et al., 1998), the levels of leakage were significantly higher in both irAOX lines compared to WT plants at 24, 48, and 72 h after \( Pst \) DC3000 infiltration (Fig. 8B).

To characterize \( Pst \)-induced cell death at a molecular level, the expression of a tobacco hypersensitive response marker \( \text{HAIRPIN-INDUCED 1 (HIN1)} \) gene was examined. \( HIN1 \) is known to be highly expressed during incompatible plant-pathogen interactions in tobacco (Takahashi et al., 2004a; 2004b). \( \text{NaHIN 1} \) transcripts were significantly induced by \( Pst \) DC3000 in \( N. \text{attenuata} \) and they were significantly higher in two irAOX lines compared to WT plants 3 d after infection (Fig. 8C). Accelerated cell death process only slightly decelerated bacterial growth in irAOX leaves at 1 (irAOX-200, 203) and 2 (irAOX-203) d after inoculation with the pathogen (Supplemental Fig. S8A).

**irAOX plants accumulate higher levels of hydrogen peroxide after \( Pst \) infection**

Cellular ROS is an important signaling molecule in eukaryotic cells (Rhoads et al., 2006). One of the proposed functions of AOX in abiotic stress resistance is the prevention of over-reduction of mitochondrial ubiquinone (UQ) pool that can counteract the formation of mitochondrial ROS (Maxwell et al., 1999; Umbach et al., 2005).

We first examined the levels of hydrogen peroxide (\( \text{H}_{2}\text{O}_{2} \)) by semi-quantitative histochemical DAB staining of WT and irAOX leaves after \( Pst \) DC3000 infection. A strong brown precipitate of oxidized DAB was observed in both WT and two irAOX lines at 1-3 d after inoculation but the staining intensity at 2 d appeared stronger in
irAOX compared to WT leaves (Fig. 9A). In order to determine H$_2$O$_2$ contents more precisely, we used sensitive quantitative Amplex Red hydrogen peroxide assay kit and determined H$_2$O$_2$ levels in the leaves. Pst DC3000 infiltration resulted in a strong increase in H$_2$O$_2$ content at 2 DPI in both WT and irAOX plants, and these levels were significantly higher in irAOX leaves (Fig. 9B), coinciding with a more rapid development of cell death symptoms in these plants (Fig. 8A).

Previously, the Arabidopsis respiratory burst oxidase homolog D (RbohD) was shown to produce a majority of ROS found in pathogen-infected Arabidopsis leaves (Torres et al., 2002). We therefore examined the expression of a putative NaRbohD homolog in N. attenuata after Pst DC3000 inoculation. The transcripts of this NaRbohD gene increased both in WT and irAOX (lines 200 and 203) but appeared significantly higher in irAOX infected leaves (Fig. 9C). This suggests that this RbohD homolog may contribute to the higher ROS levels observed in irAOX lines, possibly in response to a retrograde mitochondrial signal and/or the accumulation of SA, as proposed below.

**SA levels increase significantly more in pathogen-infected irAOX leaves**

Accumulation of SA is one of the critical features in plant defense against pathogens (reviewed by Vlot et al., 2009). As expected, SA levels dramatically increased in both WT and irAOX plants after Pst DC3000 infection, showing maximum accumulations at 24 h in Pseudomonas-infected WT leaves (Fig. 10A). SA levels steadily increased in irAOX plants and reached their putative maximum at 72 h which was 2-times higher compared to SA levels in WT plants (Fig. 10A). Interestingly, higher levels of SA in pathogen-infected irAOX plants negatively correlated with lower levels of induced JA in these leaves, suggesting a negative crosstalk between Pst DC3000-induced SA and JA accumulations (Fig. 10A).

In agreement with the higher SA levels, transcripts of two known PHENYLALANINE AMMONIA-LYASE (PAL) genes in N. attenuata, NaPAL1 and NaPAL2, were significantly more induced in irAOX compared to WT plants, attaining approximately 3-fold higher levels 72 h after inoculation (Fig. 10B).
provides a potential explanation for higher accumulation of SA in irAOX plants via benzoic acid-dependent SA biosynthetic pathway (Vlot et al., 2009). In contrast to PAL expression, the transcripts of isochorismate synthase (ICS; Vlot et al., 2009) encoding another SA biosynthetic pathway, although induced by pathogen, were not differentially regulated in irAOX and WT plants (Supplemental Fig. S8B). Similar to Empoasca spp.-attacked plants, higher levels of SA in irAOX plants were associated with the lower accumulation of JA-dependent HGL-DTGs (Supplemental Fig. S9). In contrast, the levels of a typical PAL-dependent phenolic compound, chlorogenic acid (CGA), increased in WT and irAOX plants (Supplemental Fig. S9) and this increase was pronounced in plants that lacked AOX gene function.

Discussion

Activation of AOX is an important marker of stress-adaptive cell reprogramming of plants (reviewed by Arnholdt-Schmitt et al., 2006). It is also one of the best known examples of mitochondrial retrograde regulation (MRR; reviewed by Rhoads and Subbaiah, 2007) when nuclear-encoded genes like AOX or antioxidant enzymes are induced in response to perturbation of mitochondrial homeostasis. In order to understand the role of AOX and MRR in defense of N. attenuata against biotic stress, we examined NaAOX function in three biotic stress situations using AOX-silenced tobacco plants. The NaAOX transcript and protein levels significantly increased in all cases; however, the lack of AOX transcripts in irAOX plants showed case-specific modifications of plant responses to biotic stress.

AOX-modulated responses to abiotic stress

In contrast to the well-known role of AOX in the production of heat in thermogenic plants (Meeuse, 1975), the role of the AOX in non-thermogenic plants remains elusive. Fiorani et al. (2005) observed smaller and larger plants in AOX1a antisense and sense transformed Arabidopsis plants, respectively, which were maintained at a low temperature (12°C). In an independent experiment, silencing of
the cold-inducible AOX gene did not result in any visible growth phenotype, however, several antioxidant defense genes were strongly up-regulated in the cold-exposed aox1a mutant plants, pointing to stronger cold-induced oxidative stress in these plants (Watanabe et al., 2008). Under low nitrogen supply, aox1a plants showed slight increase in gene expression of antioxidant enzymes (Watanabe et al., 2010). In tobacco plants with antisense-targeted AOX gene, higher amounts of H₂O₂ were produced under cold stress (Zhang et al. 2009). From these examples, the control of redox status and ROS levels in plants seems to be a unifying stress-related function of AOX in abiotic stresses. As discussed further, similar concept can be applied to plants under biotic stress.

**Reported roles of AOX in plant resistance to biotic stress**

To further complement the role of AOX in stress responses, the role of AOX in resistance against two types of herbivores and one pathogen was examined in native tobacco plants. Previously, the role of AOX in response to biotic stress was described in tobacco plants (Xanthi nn genotype) infected with tobacco mosaic virus (TMV). While the pretreatment of tobacco leaf discs with SA suppressed TMV replication, application of AOX inhibitor salicylhydroxamic acid (SHAM) together with SA counteracted the effect of the hormone on TMV (Chivasa et al., 1997). In addition, application of inhibitors of the cytochrome pathway (KCN or antimycin) that elicit AOX transcription via MRR, displayed levels of resistance to TMV that were comparable with SA-induced resistance (Chivasa and Carr, 1998), suggesting a role of SA as potential integrator of mitochondrial function, AOX expression and MRR. Recently, a nitric oxide (NO)-dependent systemic activation of AOX in tomato inoculated with TMV was reported by Fu et al. (2010), showing that AOX is essential for reduced accumulation of viral mRNA and systemic basal resistance to TMV in tomato plants (Fu et al., 2010). In contrast, the existence of SA-dependent and AOX-independent resistance to viruses in tobacco plants was proposed by Gilliland et al. (2003). In addition, the overexpression of AOX in transgenic *Nicotiana benthamiana* plants infected with potato virus X (PVX) increased susceptibility of
plants to systemic disease induction and virus accumulation, and AOX modulated SA-induced resistance to PVX (Lee et al., 2011).

**AOX and herbivory in N. attenuata**

Here we show that AOX transcription is efficiently induced by the feeding of a chewing herbivore. However, the suppression of AOX in irAOX plants did not impair resistance of transgenic plants to *M. sexta*, suggesting that these JA-mediated responses are robust and independent of AOX expression. In contrast, the irAOX plants interacting with piercing-sucking insect *Empoasca* spp. (and *Pst* DC3000) showed increased SA levels, suggesting that SA could act as an important intermediate between AOX-regulated processes and JA-mediated defense responses in plants that are attacked by insects associated with minimal tissue damage and/or low JA accumulations.

Even at low concentrations, SA induced transient accumulations of AOX protein and transcripts in cultured cells (Norman et al., 2004), presumably due to the interference with normal mitochondrial function. It has been shown that SA at concentrations as low as 20 μM can rapidly inhibits both ATP synthesis and oxygen uptake in the plant cells; however, the site of SA action has not been clearly elucidated (Xie and Chen, 1999). The effect of SA on respiration by intact cells and isolated mitochondria from tobacco suspension cell cultures was therefore reexamined by Norman et al. (2004). In these experiments, SA stimulated ADP-limited electron transport at less than 1 mM concentrations, acting as an uncoupler. At higher concentrations, between 1 and 5 mM, SA inhibited respiration in isolated mitochondria by preventing electron flow from the substrate dehydrogenases to the UQ pool, being a potent inhibitor of electron transport. Both the uncoupling and inhibitory effects of SA can explain lower ATP levels in cell observed by Xie and Chen (1999).

Because some of the negative effects of SA were substantially reduced in the presence of strong antioxidant N-acetylcysteine (Xie and Chen, 1999), ROS was likely involved in these responses. Because SA strongly induces AOX (Chivasa et al.,
a self-regulated interaction network involving SA, AOX and ROS may be proposed in plants. The Arabidopsis *aox1a* mutants showed enhanced ROS production in the roots after direct inhibition of Cyt respiratory pathway by KCN (Umbach et al. 2005). The lack of AOX function moderately increased H$_2$O$_2$ levels in TMV-inoculated antisense AOX-targeted tobacco plants, providing a biologically-relevant connection from SA accumulation (typical metabolite induced by TMV; Yalpani et al., 1991) to AOX-controlled ROS accumulation, similar to that we found in *Pst* DC3000-infected irAOX *N. attenuata* plants.

### Possible mechanisms of accelerated PCD in irAOX plants

Mitochondria represent one of the most important sources of ROS in plant cells. Recently, it has been shown that mitochondrial complex II of the electron transport chain is a key contributor to localized ROS accumulation during plant stress and defense responses (Gleason et al., 2011). Potato cells treated with beta-glucan elicitor displayed dramatic burst of H$_2$O$_2$, disruption of mitochondrial membrane potential and PCD when the function of catalase and AOX were simultaneously inhibited by 3-amino-1,2,4-triazol and SHAM, respectively (Mizuno et al., 2005); AOX was therefore essential for counteraction of PCD. The application of harpin elicitors strongly induced expression of Arabidopsis AOX1a (Krause and Durner, 2004), further pointing to involvement of AOX in ROS and PCD regulation. In another experiment, harpin treatment rapidly inhibited ATP synthesis in tobacco cells, similar to the effect of direct SA application (Xie and Chen, 1999), which induced PCD (Xie and Chen, 2000). Additionally, tobacco cells lacking AOX by antisense suppression of the gene showed increased sensitivity to PCD-inducing agents such as H$_2$O$_2$, SA and protein phosphatase inhibitor cantharidin (Robson and Vanlerberghe, 2002).

At the whole plant level, treatment of *Arabidopsis* leaves with SA increased the accumulation of H$_2$O$_2$, lipid peroxidation and oxidative damage to proteins (Rao et al., 1997); however, direct application of H$_2$O$_2$ failed to mimic these events. Taking into account the effects of SA on mitochondria, SA is very likely to control mitochondrial
ROS in concert with AOX to fine-tune the progression of PCD in plants, supported by our findings in *N. attenuata* plants challenged with *Pst* DC3000. The expression of Hsr203J, a known PCD marker in tobacco (Takahashi et al., 2004b) increased significantly more in irAOX leaves compared to WT after *Pst* infection, supporting our view that AOX functionally counteracts the early development of PCD in pathogen infected leaves. Recently, Kiba et al. (2008) reported enhanced cell death in *Pseudomonas*-infected lettuce leaves after octyl gallate treatment, an effective inhibitor of AOX.

**AOX affecting JA-SA hormonal crosstalk**

In our experiments with piercing-sucking insects, irAOX plants became more susceptible to leafhopper feeding, both in the glasshouse and under Utah field conditions. The greater susceptibility to piercing-sucking insects correlated with higher accumulation of SA that was associated with a lower content of late-accumulating JA-dependent defense metabolites in irAOX plants. Recently, a detailed analysis of *Empoasca* host choice was conducted, revealing a key role of JA in initial determination of plant host by these insects (Kallenbach et al. 2012); *Empoasca* spp. damage negatively correlated with the ability of these plants to perceive wound-induced jasmonates (Kallenbach et al., 2012). Similarly, the ectopically higher levels of SA in irAOX plants at later time points could interfere with downstream JA signaling in *N. attenuata* plants, resulting in enhanced feeding of leafhoppers on these plants. Recently, Leon-Reyes et al. (2010) showed that SA strongly inhibits jasmonate-mediated gene expression in Arabidopsis, leading to a significant down-regulation of typical JA-responsive genes like *PDF1.2* and *VSP2* by mechanisms located downstream of JA biosynthesis. Although we did not find directly reduced JA levels in *Empoasca* spp.-attacked irAOX leaves (associated with moderate AOX-dependent increase of SA), we found significantly reduced JA levels in *Pseudomonas*-infected plants (associated with greatly elevated SA levels). Remarkably, lower levels of JA-dependent defense-related HGL-DTGs were identified in both interactions. These data suggest that the amounts as well as ratios
between SA and jasmonates may be important to determine the outcome of stress-modulated JA-SA crosstalk in plants.

Insects frequently serve as vectors/hosts for microbial pathogens that cause diseases in plants (Weintraub and Beanland, 2006). Although leafhopper-associated Candidatus Phytoplasma spp. have not been detected in our insect colony or from insects collected in the field (Kallenbach et al., 2012), other leafhopper-transmitted microbes, such as viruses or bacteria, could have been responsible for ectopic accumulation of SA and activation of SA-dependent signaling in AOX-deficient N. attenuata plants. Plant anti-herbivore defenses countered by pathogenic infections associated with higher levels of SA were recently reported by Thaler et al. (2010). The effect of TMV infection on herbivore performance was tested in WT and SA-deficient (NahG) plant backgrounds, showing that SA was required for TMV to increase herbivore vector performance.

Priming and activation of plant immune responses triggered by some beneficial microbes point to an existence of complex interactions mediating both, positive and negative, roles of microbes in ecological interactions (reviewed by Van Wees et al., 2008). It was previously shown that MeSA derived from SA is required for systemic acquired resistance (SAR) signal perception in systemic tobacco tissues (Park et al., 2007). In the same study, it was proposed that a lipid-derived SAR signal could work with (or upstream of) MeSA to activate SAR. Indeed, the local production of MeSA from SA in Arabidopsis by methyltransferase enzyme was shown to be dependent on the P. syringae virulence factor coronatine (functional analog of JA-Ile) (Attaran et al., 2009). Because most MeSA was directly emitted into the atmosphere, and only a small amount was retained in the leaves, it was proposed that the phytopathogen is using JA signaling-dependent, coronatine-mediated volatilization of MeSA from the leaves to attenuate the SA-based defense pathway.

Although antagonistic interactions prevail in examples of JA-SA crosstalk, the effects of both signaling pathways need not be mutually exclusive when these responses become temporally or spatially separated. Several JA perception, biosynthetic and signaling mutants were attenuated in RPM1-mediated systemic
immunity (SAR) in Arabidopsis plants challenged with an avirulent strain of Pseudomonas syringae, consistent with a positive role of JA in systemic immunity (Truman et al., 2007). In addition, it was noticed that SA often accumulates 1 day after JA accumulation in natural plant’s innate-immune-response to viruses (Shang et al., 2011), suggesting that JA and SA can act in synergism and confer optimal virus resistance when applied in appropriate concentrations and with a time delay. Indeed, when these changes were simulated by exogenous application of JA and SA, the strong inhibitory efficiency to virus replication was achieves (80–90%). It will be interesting to see in the future if AOX could be directly involved in these interactions.

**Conclusions**

Although AOX expression is induced by numerous biotic stress factors, the induction of AOX and protection against stress may not always be in a positively linear relationship. *N. attenuata* plants clearly benefited from the activity of AOX and alternative respiration to cope with leafhopper attack, and controlled progression of PCD during *Pseudomonas* infection. However, they did not gain any clear benefit from inducing AOX after being attacked by *M. sexta* caterpillars. These results prompt further investigations and the use of additional plant-biotic stress models to unravel additional ecological and physiological roles of alternative respiratory pathway in plants.

**Materials and methods**

**Plant growth and treatments**

Wild-type *N. attenuata* plants (22nd inbred generation) seeds, originally collected from a native population in Utah, USA, were used for all experiments, including transformation and generation of transgenic lines. Wild-type plants were transformed with constructs carrying fragments in an inverted repeat orientation of *N. attenuata* alternative oxidase 1 (AOX1; AY422688) and lipoxygenase-3 (LOX3) which mediates JA production (Halitschke and Baldwin, 2003). All transformed plants were
diploid as determined by flow cytometry and homozygous for a single transgene insertion. Plants from T2 or T3 generation were used in experiments. Seeds were germinated on agar plates supplemented with Gamborg B5 media (Duchefa) according to the procedures described by (Krügel et al., 2002). All treatments were performed on rosette stage plants grown in 1L pots with soil, unless otherwise noted. For W+W and W+OS treatments, leaves were wounded with a pattern wheel and 20 µL of water or *M. sexta* OS (one-fifth diluted in water) was rubbed into wounds, respectively. For *M. sexta* feeding assays, two freshly hatched neonate larvae were placed on the leaves of each WT and irAOX plants. Clip cages were used to restrict movement of larvae on the plant and samples were collected after 0, 1, 2, and 3 d. Clip-caged leaves from separate plants without larvae were used as controls. For *Empoasca* spp. feeding assays, WT and transgenic lines were placed into a glass container (110x50x80 cm) with *Empoasca* spp. leafhoppers reared on *Cucurbita moschata, C. maxima* (var. “Goldnugget”) and *C. pepo* plants, and leafhoppers were allowed to choose the host plants. The estimated colony size at the time of experiment was 800-1000 leafhoppers in the glass box. Samples from *Empoasca* spp.-infested plants were collected after 0, 4, 8, and 12 d. A set of plants growing next to the box with *Empoasca* spp. were used to collection control samples. Bacterial strain of *Pseudomonas syringae* pv. tomato DC3000 was cultivated on Luria-Bertani (LB) plates supported with agar containing 25 mg/L rifampicin and 5 mg/L tetracyclin at 28°C. Bacterial growth assays and inoculation of the leaves were carried out essentially as described in Rayapuram et al. (2008).

In the field, 15-day-old seedlings were transferred into previously hydrated 50-mm peat pellets (Jiffy 703, [http://www.jiffypot.com](http://www.jiffypot.com)) grown in shade tents for two weeks to gradually adjust the plants to the high sun and low relative humidity conditions of the Great Basin Desert. Three to four weeks later, when plants were in the early-rosette stage of growth, size-matched WT/irAOX-203 plant pairs were transplanted into an irrigated field plot (at a distance of 1.5 m between the pairs) in the 2008 growing season at the Lytle Ranch Preserve, near Santa Clara, Utah. The release of transgenics was carried under APHIS notification 06-242-3r and the seeds
were imported under 07-341-101n.

**Generation and characterization of NaAOX-silenced plants (ir-AOX)**

A 510 bp fragment of the cDNA sequence of *NaAOX1* gene (position 338-848 in AY422688) was inserted into the pSOL3 transformation vector as an inverted-repeat construct to generate pSOL3AOX vector (Supplementary Figure S3A). *N. attenuata* WT plants were transformed with pSOL3AOX vector using an *Agrobacterium*-mediated transformation procedure previously described by Krügel et al. (2002). The gene for hygromycin resistance (hptII) allowed selecting hygromycin-resistant transgenic plants on the agar plates supplemented with 35 mg/L hygromycin (Krügel et al., 2002). Southern blot hybridization of genomic DNA from independently transformed T1 generation plants and from WT line was carried out according to a standard protocol, using 32P-labeled PCR fragment of hptII gene as a probe. Independent transgenic lines harboring a single copy of the transgene (Supplementary Figure S3B) were further screened for homozygosity after germination on hygromycin-containing GB5 media. Two independently transformed single-insert homozygous lines 200 and 203, strongly suppressed in their *NaAOX* transcript accumulation, were selected for further experiments.

**Expression analysis by RT-qPCR**

Total RNA was extracted from approximately 100 mg leaf tissue using TRIZOL reagent ([Invitrogen](http://www.invitrogen.com)) and total RNA was treated with RQ1 RNase-free DNAse to remove all DNA contaminants ([Promega](http://www.promega.com)). RNA samples were diluted to a final concentration 0.5 µg/µL and 1 µg of total RNA was used for cDNA synthesis with oligo-dT primer ([Fermentas](http://www.fermentas.com)) and Superscript II reverse transcriptase ([Invitrogen](http://www.invitrogen.com)) following a standard protocol. RT-qPCR assays were performed on a Stratagene Mx3005P real-time PCR system ([http://www.stratagene.com](http://www.stratagene.com)) using qPCR kit for SYBR Green I and qPCR Core kit for Taqman assays ([Eurogentec GmbH](http://www.eurogentec.com)). To determine
NaAOX gene transcript levels in irAOX lines, gene specific primers were designed outside the region used in the ir silencing construct. Gene-specific primers were designed with Primer3 software (http://frodo.wi.mit.edu/primer3). Relative gene expression was calculated from linear standard curves prepared by serial dilutions of a specific cDNA standard sample. Actin house-keeping gene from *N. attenuata* (EU273278) was used as endogenous reference to normalize the gene expression. The primers, Taqman probe sequences, and primer sequences for SYBR Green-based RT-qPCR are described in Supplemental Table 1.

**AOX protein determination by western blotting**

Leaves were ground to a fine powder in liquid nitrogen and 100µL of 2x Laemmli buffer with 2% (v/v) β-mercaptoethanol was added to 150 mg tissue before protein extractions. The samples were centrifuged for 20 min at 16,100 x g and protein concentration in the cleared supernatants was determined by the Bradford reagent (Sigma; http://www.sigmaaldrich.com). The aliquots (25 µg) of protein were boiled for 3 min in Eppendorf tubes and separated on 10% SDS-PAGE gel. Proteins from the gel were electroblotted onto a PVDF membrane (GE Healthcare Life Sciences http://www.gelifesciences.com) in Tris-Gly buffer and 20% (v/v) methanol. AOX protein was immunodetected using a commercial polyclonal antibody against *Arabidopsis thaliana* AOX (Agrisera http://www.agrisera.com) at a dilution of 1:1000 and WesternBreeze® immunodetection kit (Invitrogen http://www.invitrogen.com). The manufactures’ instructions were followed except in the following modifications: membranes were blocked overnight at 4 °C and washing steps were prolonged to 10 min.

**Measurement of respiration in *N. attenuata* leaves**

The plants were placed in dark for 15 min before the start of respiration measurements. Leaf discs were excised by 8 mm diameter cork borer from the leaves and placed in in the plastic syringe with assay buffer (20 mM Hepes, 0.2 mM CaCl2, pH 7.2; Simons et al. 1999) supplied with inhibitors or without inhibitors for
determination of total respiration. The leaf discs were briefly infiltrated by creating a partial vacuum in the syringe and incubated in the dark for 15 min. Four infiltrated discs (approximately 80 mg FM) were transferred into 4 mL glass vial filled with fresh O₂-saturated assay buffer (2 mL) equipped with a miniaturized Clark-type oxygen electrode (Oxygen Microsensor, Unisense, Denmark). After initial equilibration of the electrode for several minutes, O₂ consumption was continuously measured in dark for 2-3 min and recorded using Sensor Trace Basic 3.1.1 software (Unisense, Denmark) in 10 sec intervals. Total respiration rates (V_{TOTAL}) were determined as leaf disc O₂ uptake (μmol min⁻¹ g FM⁻¹) in the assay buffer without inhibitors. The cyanide-sensitive cytochrome c dependent respiration (V_{CYT}) was determined in the assay buffer containing 1 mM KCN and calculated as V_{TOTAL} – V_{KCN}. Finally, cyanide-insensitive but SHAM-sensitive alternative respiration (V_{ALT}) was determined after addition of 5 mM SHAM to 1 mM KCN-containing buffer and calculated as difference V_{KCN} – V_{KCN+SHAM} (Furuhashi et al. 1989).

**Phytohormone analysis**

Harvested leaves were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Phytohormones SA, JA and JA-Ile were extracted following the procedure described in Wu et al. (2007). In brief, 150 mg of leaf material was extracted with 1 mL of ethyl acetate spiked with 200 ng D₄-SA, ¹³C₂-JA, and ¹³C₆-JA-Ile as internal standards in FastPrep tubes containing 0.9 g of FastPrep Matrix (Sili GmbH; http://www.sigmund-lindner.com). The tissues were homogenized in 2000 GENO/GRINDER homogenizer (SPEX CertiPrep; http://www.spxcsp.com) for 2 min and samples were centrifuged at 13,000 x g for 20 min at 4 °C. The supernatants were transferred to clean 2-mL Eppendorf tubes and each pellet was re-extracted with 1 mL of ethyl acetate and centrifuged. Combined supernatants were dried in a vacuum concentrator to near dryness (Eppendorf; http://www.eppendorf.com) before re-suspending the samples in 0.5 mL of 70% methanol (v/v). A 10 μL of extract was subjected to reverse-phase HPLC coupled to tandem mass spectrometry using 1200LC LC/MS-MS-MS system (Varian, Palo Alto,
CA, USA; http://www.varianinc.com) according to the procedure described in Wu et al. (2007). The levels of each phytohormone were calculated based on the peak area of the labeled internal standards and FM of the sample.

**Analysis of secondary metabolites**

Secondary metabolites were analyzed by HPLC coupled to diode array detector (DAD) as described in Kaur et al. (2010). Approximately 100 mg of leaf material was extracted with 1 mL of extraction buffer containing 40% methanol and 0.5% acetic acid. After centrifugation, the supernatants were collected and injected into an Agilent 1100 series HPLC (http://www.chem.agilent.com) equipped with ODS Inertsil C18 column (3 μm, 150 x 4.6 mm i.d.) that was protected by a Phenomenex Security Guard C18 pre-column (Phenomenex; http://www.phenomenex.com). Nicotine, rutin, and chlorogenic acid (CGA) standards were used to construct corresponding calibration curves. CP and DCS contents were calculated on the basis of CGA calibration that showed nearly identical UV-absorption profile.

**Determination of cell death**

Trypan blue staining specific to dead cells with permeabilized cytoplasmic membranes was carried out as described in Koch et al. (1990). Electrolyte leakage was measured as described in Pike et al. (1998) using a conductivity meter (Mettler Toledo, Ohio; http://us.mt.com/). Eight discs (0.785 cm² each) were punched from the treated leaves using a cork-borer and placed in 15 mL Falcon tubes (BD Biosciences http://wwwbdbiosciences.com) together with 10 mL of sterile distilled water. Samples were incubated for 4 h under slow rotation at 20 °C and the conductivity of resulting water solutions was measured by conductivity meter.

**Hydrogen peroxide visualization and determinations**

DAB staining was performed on the leaves inoculated with Pst DC3000 or mock solutions as described by Thordal-Christensen et al. (1997) with some modifications. Leaves with petioles were excised from the plants at each time point after bacteria
infiltration and supplied with 1mg/mL DAB solution through the petioles until a brown precipitate was observed in the leaf blades (5 h). Leaves were de-stained in boiling ethanol (96%) for 10 min and fixed in 3:1:1 ethanol/lactic acid/glycerol solution. To determine endogenous concentrations of H$_2$O$_2$, liquid nitrogen-stored leaves were ground in liquid nitrogen and 100 mg of tissue powders were extracted in 200 µL of 25 mM HCl supplemented with 50 mg of activated charcoal. After 1-2 min vortexing, samples were kept on ice for 10 min and centrifuged at 12000 x g for 20 min at 4 ºC. Cleared supernatants were transferred to clean tubes and H$_2$O$_2$ concentrations were determined using Amplex red H$_2$O$_2$/peroxidase assay kit (Invitrogen; http://www.invitrogen.com). H$_2$O$_2$ was used to construct calibration curves. Prior to each measurement, 10 µL of each extract was neutralized with 90 µL of 1x reaction buffer and 50 µL of neutralized extract was loaded on a 96 well Nunc multiwell plate (Thermo Scientific; http://www.nuncbrand.com). Next, 50 µL of reaction buffer was dispensed into each well to initiate the reaction. Samples were incubated for 30 min at RT before measurement of fluorescent intensities at Excitation/Emission 530/590 nm in an Infinite 200 multimode reader (Tecan Group Ltd.; http://www.tecan.com).

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Figures and legends

**Figure 1**

*NaAOX* gene transcripts increase in response to simulated herbivory and feeding of *M. sexta* caterpillars in *N. attenuata*. (A) Technical replicate means (± SE) of *NaAOX* transcript relative abundances quantified with RT-qPCR in pooled samples using 5 independent control, W+W, and W+OS-elicited *N. attenuata* plants. The leaves were elicited with a pattern wheel (wounding) and immediately applying either water to mechanical wounds (W+W) or *M. sexta*’s oral secretions (OS) to simulate herbivory (W+OS). Gray areas indicate dark periods
showing lower NaAOX transcript levels. (B) Means (±SE) of NaAOX transcripts quantified with RT-qPCR from control and M. sexta-attacked rosette leaves at 0, 1, 2, and 3 d after placing 3 M. sexta neonates on each leaf. Asterisks indicate significant differences between control and insect-fed plants at respective time points (unpaired Student’s t-test, *P<0.05; n=3).

Figure 2
irAOX OS- and herbivory-induced plants have WT jasmonate levels and AOX silencing does not affect M. sexta caterpillar performance. (A) Mean (± SE) levels of JA and JA-Ile in samples collected at 0, 0.75, 1.5, and 3 h after wounding and immediately applying OS from M. sexta (W+OS, n=5, left panel), and mean (± SE) levels of JA and JA-Ile in samples collected after 0, 1, 2, and 3 d of M. sexta feeding (n=3, right panel). (B) Mean (± SE) mass of M. sexta caterpillars determined after neonates placed on 30 independent WT and irAOX plants were allowed to feed for 12 d. No statistically significant differences in caterpillar mass were detected between WT and NaAOX gene-silenced lines.

Figure 3
irAOX plants are more susceptible to Empoasca leafhopper damage in the glasshouse and natural environment. (A) Mean (± SE) average leaf damage by Empoasca spp. in WT and irAOX plants planted in a pair-wise design in the natural environment of N. attenuata in Great Basin Desert (n=25). (B) Leaves from WT and irAOX plants that were placed for 12 d in a closed-cage in the glasshouse with Empoasca spp. leafhoppers (left), and mean (± SE) leaf damage by Empoasca spp. feeding estimated at the end of experiment (n=10, right). Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by unpaired Student’s t-test (*P<0.05; ***P<0.001). Inset in (A): Empoasca spp. photographed in the field.

Figure 4
Leaf NaAOX gene transcript levels increase in response to Empoasca spp. leafhopper attack. Means (±SE) of NaAOX relative transcript abundances quantified with RT-qPCR in
control and *Empoasca* spp.-attacked WT leaves at 0, 4, 8, and 12 d. Asterisks indicate significant differences between control and *Empoasca* spp.-damaged plants at respective time points (unpaired Student’s *t*-test. **P<0.01, ***P<0.001; *n=6*).

Figure 5

**Long term *Empoasca* spp. feeding results in differential SA but not jasmonate levels in irAOX plants.** (A) Mean (± SE) levels of SA and (B) JA and JA-Ile in samples collected after 0, 4, 8, and 12 d of *Empoasca* spp. feeding. Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by unpaired Student’s *t*-test (*P<0.05, **P<0.01; *n=6*).

Figure 6

**Secondary metabolite levels significantly differ in *Empoasca* spp. attacked WT and irAOX leaves.** Mean (± SE) levels of defensive secondary metabolites in WT and irAOX-203 leaf samples collected after 0, 4, 8, and 12 d of exposure to *Empoasca* spp. Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by unpaired Student’s *t*-test (*P<0.05; **P<0.01; *** P<0.001; *n=6*).

Figure 7

**NaAOX gene transcripts increase in response to infection with Pst DC3000 in N. attenuata leaves.** Mean (±SE) NaAOX relative transcript abundances quantified with RT-qPCR in 3 independent mock- and Pst pv. tomato DC3000-inoculated WT plants at indicated time points. Asterisks indicate significant differences between mock- and Pst DC3000-infected leaves at respective time points determined by unpaired Student’s *t*-test (*P<0.05; **P<0.01; *** P<0.001; *n=3*).

Figure 8

**NaAOX gene silencing accelerates cell death after infection of plants with Pst DC3000 pathogen.** (A) Pst DC3000-infected leaves from WT and two irAOX independent lines (200
and 203) at 1, 2, and 3 d after inoculation (left panel), and trypan blue stained leaves at the corresponding time points (right panel). (B) Mean (± SE) electrolyte leakage from mock and Pseudomonas-infected leaves in WT and two irAOX lines determined at 1, 2, and 3 d after inoculation. Significant differences were determined for each time point by one-way ANOVA (*P<0.05; **P<0.01; *** P<0.001; n=5). (C) Mean (±SE) relative transcript levels of cell death marker gene NaHIN1 in mock and Pst DC3000-infected leaves of WT and two irAOX lines. Significant differences at respective time points were determined by one-way ANOVA (*P<0.05; **P<0.01; n=5).

Figure 9

Pseudomonas-infected leaves of irAOX plants show higher levels of hydrogen peroxide and increased expression of the NaRbohD gene. (A) DAB-stained Pst DC3000-infected leaves from WT and two irAOX lines at 1, 2, and 3 d post inoculation. (B) Mean (± SE) hydrogen peroxide levels in mock- and Pst DC3000-infected WT and irAOX leaves determined at 24 and 48 h post inoculation. Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by unpaired Student’s t-test (**P<0.01, n=5) (C) Mean (± SE) of NaRbohD relative transcript abundances quantified with RT-qPCR in mock- and Pst DC3000-inoculated leaves at indicated time points (n=3). Asterisks show significant differences between WT and irAOX plants at respective time points determined by one-way ANOVAs (*P<0.05; **P<0.01; *** P<0.001).

Figure 10

NaPAL gene transcript accumulation correlates with increased SA accumulation in Pst DC3000-infected irAOX leaves. (A) Mean (± SE) levels of SA and JA in WT and irAOX leaves determined 0, 24, 48, and 72 h after Pst DC3000 infection (n=5). Note large difference in scale between mock and Pst DC3000-inoculated SA levels. (B) Means (±SE) of NaPAL relative transcript abundances quantified with RT-qPCR in mock and Pst DC3000-infected WT and two irAOX lines at indicated time points (n=3). Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by one-way ANOVA (*P<0.05; ** P<0.01).
Supplemental figures

Supplemental Figure S1

AOX genes from *N. attenuata* are highly homologous to AOX1a gene from *Arabidopsis thaliana*. (A) Deduced NaAOX1 (AY422688) and NaAOX2 (AY422689) protein sequences were aligned with AtAOX1a protein from *A. thaliana* (AT3G22370.1). Asterisks show conserved amino acids in all three proteins. (B) The accumulation of AOX transcripts in local treated leaves was determined by microarrays (*n*=3) after elicitation of the leaves with wounding (W+W) or simulated herbivory (W+OS); control plants remained untreated. Control and W+OS samples were harvested at 0.5, 1, 5, 9, 12, 17 and 21 h post treatment; samples from W+W treatment were collected at 1, 5 and 17 h post treatment.

Supplemental Figure S2

*NaAOX* proteins increase in response to simulated herbivory, feeding of *M. sexta* caterpillars, *Empoasca* spp. attack and *P. syringae* DC3000 infection in *N. attenuata*. (A) NaAOX protein levels after simulated herbivory as shown in Fig. 1A determined by western blotting with specific antibody raised against conserved AOX protein peptide sequence; (B) AOX levels after *M. sexta* caterpillar feeding as in Fig. 1B; AOX levels after *Empoasca* spp. attack as in Fig. 4; and (D) AOX levels during *Pst* infection as in Fig. 7. *rbcL* shows the RuBisCo large subunit (~50 kDa) in a Ponceau S-stained membrane prior to western blotting (reddish pink stain).

Supplemental Figure S3

Structure of plant transformation vector pSOL3AOX used for silencing of *AOX* genes in *N. attenuata* and characterization of irAOX transgenic lines. (A) A 511bp of *NaAOX1* gene (position 338-848 in AY422688) was inserted into pSOL3 vector as an inverted-repeat construct with *hptII* used as plant selection marker gene. (B) Two *Agrobacterium*-transformed
lines irAOX-200 and irAOX-203 were subjected to Southern blot analysis using genomic DNA digested with XbaI restriction enzyme and hptII-radiolabeled probe. Both lines showed single insertion of T-DNA fragment into the genome. (C) Mean (± SE) levels of NaAOX transcripts in unelicited and 0.75h W+OS-elicited leaves of two independently transformed homozygous irAOX lines and wild-type plants. Asterisks indicate significant differences between WT and individual irAOX lines (one-way ANOVA, *** P<0.001; n=5). Note the break in the Y-axis.

Supplemental Figure S4

**Functional characterization of irAOX lines.** Oxygen consumption in the leaves was determined with a miniaturized oxygen Clark-type electrode (Oxygen microsensor, Unisense, Denmark) after incubation of leaf discs in oxygen-saturated buffer and application of (A) no inhibitors to determine total respiration (B) 1 mM KCN to determine cyanide-sensitive cytochrome c respiration, (C) 1 mM KCN and 5mM SHAM to determine SHAM-sensitive alternative respiration. (D) irAOX plants, supported by efficient silencing of AOX genes shown in Supplemental Figure S3C, showed very low alternative respiration rates demonstrated as reduced portion of SHAM-sensitive respiration capacity. In contrast, irAOX plants showed higher activity of cytochrome c pathway, most likely due to reduced competition by AOX pathway and higher availability of reduced ubiquinone pool. Experiment was conducted with 3 biological replicates using WT and 2 transgenic lines, irAOX-200 and irAOX-203. Statistically significant differences were determined by one-way ANOVA (P < 0.01, n=3).

Supplemental Figure S5

**irAOX WOS- and herbivory-induced plants have salicylate levels and hydrogen peroxide (H$_2$O$_2$) levels comparable to WT plants.** (A) Mean (± SE) levels of SA in the samples collected at 0, 0.75, 1.5, and 3 h after W+W (n=5, left panel) and W+OS (n=5, right panel) treatments. (B) Mean (± SE) levels of SA in samples collected in control plants (n=3, left panel) and plants after 0, 1, 2, and 3 d of *M. sexta* feeding (n=3, right panel). (C) Intact leaves for the DAB (H$_2$O$_2$) staining were treated with W+W, W+OS or remained untreated.
(control) and they were detached from the plants 45 min after treatment. The entire excised leaf was floated in the DAB staining solution for 24 h in the dark and de-stained to visualize brown precipitate of DAB indicating the presence of H$_2$O$_2$.

Supplemental Figure S6

**Exposure to *M. sexta* feeding does not change a majority of the secondary metabolite profiles in irAOX plants compared to WT.** Mean (± SE) levels of secondary metabolites from WT and irAOX plants collected after 0, 1, 2, and 3 d of *M. sexta* feeding (*n*=3). Samples were extracted in acidified 40% methanol and subjected to HPLC separation and detection based on UV absorbance of the compounds (17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), detected at 210 nm; nicotine, 254 nm; chlorogenic acid, caffeoylputrescine, dicaffeoylspermidine, 320 nm; rutin, 360 nm).

Supplemental Figure S7

**Silencing of NaLOX3 compromises defense of *N. attenuata* against *Empoasca* spp.** Leaves from WT and irLOX3 plants with reduced JA levels after 12 d of exposure to *Empoasca* spp. leafhoppers. Numbers show mean (± SE) average leaf damage by *Empoasca* spp. estimated at the end of experiment (12 d). Asterisks indicate significant differences between WT and irLOX3 genotypes determined by unpaired Student’s *t*-test (* P<0.05; *n*=6).

Supplemental Figure S8

**Multiplication of *Pst* DC3000 is slightly suppressed in irAOX plants and *NaICS* transcript levels are not differentially regulated in WT and irAOX plants infected with *Pst* DC3000.** (A) Mean (±SE) Log$_{10}$ of colony forming units (CFU) determined in *Pst* DC3000-infected WT and irAOX leaves at indicated time points. Significant differences at respective time points were determined by one-way ANOVA (*P<0.05; **P<0.01; *n*=5). (B) Mean (±SE) relative transcript levels of *N. attenuata ISOCHORISMATE SYNTHASE* (*NaICS*) gene in mock and *Pst* DC3000-inoculated leaves of WT and two irAOX lines. No significant differences between genotypes at respective time points were determined by one-way ANOVA.
Supplemental Figure S9

Secondary metabolites levels are altered in irAOX plants relative to WT after Pst DC3000 infection. Mean (± SE) levels of secondary metabolites from samples collected 24, 48, and 72 h after infection. Significantly higher chlorogenic acid (CGA) levels correlated with the increased SA levels and higher PAL transcripts in irAOX plants, while significantly lower 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTG) levels correlated with decreased JA levels in these plants. Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by one-way ANOVA (* P<0.05; *** P<0.001; n=6).

Supplemental tables

Supplemental Table 1

Real time PCR primers and probes. The primers and Taqman probe sequences used for Taqman qPCR, and primer sequences used for SYBR Green-based RT-qPCR assays.
NaAOX gene transcripts increase in response to simulated herbivory and feeding of *M. sexta* caterpillars in *N. attenuata*. (A) Technical replicate means (± SE) of NaAOX transcript relative abundances quantified with RT-qPCR in pooled samples using 5 independent control, W+W, and W+OS-elicited *N. attenuata* plants. The leaves were elicited with a pattern wheel (wounding) and immediately applying either water to mechanical wounds (W+W) or *M. sexta*’s oral secretions (OS) to simulate herbivory (W+OS). Gray areas indicate dark periods showing lower NaAOX transcript levels. (B) Means (± SE) of NaAOX transcripts quantified with RT-qPCR from control and *M. sexta*-attacked rosette leaves at 0, 1, 2, and 3 d after placing 3 *M. sexta* neonates on each leaf. Asterisks indicate significant differences between control and insect-fed plants at respective time points (unpaired Student’s t-test, *P*<0.05, *n*=3).
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Leaf NaAOX gene transcript levels increase in response to *Empoasca* spp. leafhopper attack. Means (±SE) of NaAOX relative transcript abundances quantified with RT-qPCR in control and *Empoasca* spp.-attacked WT leaves at 0, 4, 8, and 12 d. Asterisks indicate significant differences between control and *Empoasca* spp.-damaged plants at respective time points (unpaired Student’s t-test, **P<0.01, ***P<0.001; n=6).
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**NaPAL** gene transcript accumulation correlates with increased SA accumulation in *Pst* DC3000-infected irAOX leaves. (A) Mean (± SE) levels of SA and JA in WT and irAOX leaves determined 0, 24, 48, and 72 h after *Pst* DC3000 infection (*n=5*). Note large difference in scale between mock and *Pst* DC3000-inoculated SA levels. (B) Means (± SE) of NaPAL relative transcript abundances quantified with RT-qPCR in mock and *Pst* DC3000-infected WT and two irAOX lines at indicated time points (*n=3*). Asterisks indicate significant differences between WT and irAOX plants at respective time points determined by one-way ANOVA (*P<0.05; **P<0.01*).