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Glycolate oxidase is an alternative source for H_2O_2 production during plant defense responses and functions independently from NADPH oxidase

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Abbreviations: HR, hypersensitive response; ROS, reactive oxygen species; Atrboh, respiratory burst oxidase; GOX, glycolate oxidase

The photorespiratory enzyme glycolate oxidase (GOX) was found to be involved in nonhost resistance by regulating plant defense responses through the production of H_2O_2. Silencing of a gene encoding NADPH oxidase (*AtRBOHD*) in the *gox* mutants did not further increase susceptibility to a nonhost pathogen, *P. syringae* pv tabaci, although it caused an increase in bacterial growth in the *Atgox1* and *Atgox3* mutant backgrounds. In order to confirm this finding, we created double homozygous knockouts *AtrbohD* x *Atgox1* and *AtrbohD* x *Atgox3* to evaluate symptom development and bacterial growth. Here we show that there is no additive effect of disease symptoms or bacterial growth in the *AtrbohD* x *Atgox1* and *AtrbohD* x *Atgox3* double mutants when compared with individual mutants. Slight additive effect observed previously upon silencing of *AtRBOHD* in *Atgox1* and *Atgox3* mutants was most likely due to cross-silencing of *AtRBOHF*. These results further prove that GOX plays a role in nonhost resistance independent of NADPH oxidase.

Plants are able to detect the threat of pathogens by mechanisms that include a plethora of preformed compounds as well as complex induced responses.[1] In most cases these mechanisms are enough to stop the pathogen before it is able to cause significant damage to the plant. In nature, the most common form of disease resistance is attributed to nonhost resistance defined as the resistance exhibited by entire plant species to all isolates of a microbial species."[2,3] A typical feature of nonhost resistance is a hypersensitive response (HR), a form of programmed cell death that occurs around the site where pathogens or elicitors interact with the plant.[3] This HR involves accumulation of the reactive oxygen species (ROS) like superoxide (O_2^-), and the most stable form hydrogen peroxide (H_2O_2).[4]

The source of ROS during plant defense has been controversial and for many years it has been proposed that upon pathogen recognition, ROS production occurs through the action of NADPH oxidases, also called respiratory burst oxidases (RBOH).[5] NADPH oxidase reaction uses O_2 to generate superoxide (O_2^-) which is converted into H_2O_2 by superoxide dismutase (SOD).[5] In Arabidopsis, 10 genes encode NADPH oxidases but only two, *AtRBOHD* and *AtRBOHF*, have been implicated in plant defense.[6,7] *AtrbohD* mutant was shown to be defective in the production of H_2O_2 after inoculation with the avirulent bacterium *P. syringae* pv tomato DC3000 (*AvrRpm1*) although it was not compromised in HR[6] and exhibited distinct behavior depending on the pathogen used for inoculation. Thus, the growth of *P. syringae* pv tomato DC3000 (*AvrRpm1*) was not affected in this mutant and bacteria grew to the same level as wild-type plants[8] However, the accumulation of the oomycete *Peronospora parasitica*[6] and the fungus *Alternaria brassicicola*[9] was reduced in the *AtrbohD* mutant when compared with wild-type plants. Conversely, *AtrbohF* mutant which was not defective in H_2O_2 accumulation,[6] allowed higher accumulation of the virulent bacteria *P. syringae* pv tomato DC3000 while the accumulation of the avirulent *P. syringae* pv tomato DC3000 (*AvrRpm1*) was not different than in wild-type plants.[8] Similar to *AtrbohD* mutant, there was less sporangiophore formation due to *Peronospora parasitica* infection in *AtrbohF* mutant plants.[6]

We recently showed that another source of H_2O_2 involved in defense responses against the nonhost pathogen *P. syringae* pv tabaci is the photorespiratory enzyme glycolate oxidase (GOX).[10] The generation of H_2O_2 by GOX occurs when it catalyzes the oxidation of glycolate to glyoxylate.[11] Similarly to NADPH oxidase, there are multiple genes encoding GOX in Arabidopsis: *AtGOX1*, *AtGOX2*, *AtGOX3, AtHAOX1* and *AtHAOX2*.[12] Two of these genes *AtGOX3* and *AtHAOX2* were induced by *P. syringae* pv tabaci in wild-type plants and their corresponding mutants showed significant reduction in the expression of defense genes. These results suggested that the H_2O_2 generated by GOX is used as a signal to activate several defense signal transduction pathways.[10]

In order to determine whether NADPH oxidase was involved in the phenotypes observed, we silenced *AtRBOHD* in the

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wild-type (Col-0) and the *gox* mutant backgrounds and evaluated disease development and bacterial growth.[10] Silencing of *AtRBOHD* in wild-type background slightly increased symptoms after inoculation with the nonhost pathogen *P. syringae* pv tabaci when compared with non-silenced control plants. However, *AtRBOHD* silencing in *gox* mutant backgrounds did not have an additive effect on disease symptoms after inoculation with *P. syringae* pv tabaci.[10] Interestingly, when bacterial growth was quantified, we observed a slight increase in the number of bacteria after silencing of *AtRBOHD* in *Atgox1* and *Atgox3* backgrounds that warrants further investigation.[10] In addition, *AtRBOHD* silencing in both wild-type and *gox* mutant backgrounds caused cross silencing of *AtRBOHF* and therefore the exact role of *AtRBOHD* in *gox* mutants was not clear. We therefore decided to create double mutants by crossing *AtrbohD* mutant with *Atgox1* and *Atgox3* mutants. Double knockouts of *AtrbohD* x *Atgox1* and *AtrbohD* x *Atgox3* were made and double homozygous plants were identified. For inoculations, double homozygous mutant plants were grown simultaneously with single mutant parents and with the wild type, Col-0. Six-week old plants were syringe-inoculated with the nonhost pathogen *P. syringae* pv tabaci and the host pathogen *P. syringae* pv maculicola to observe symptom development (**Fig. 1**). Inoculation with the nonhost pathogen *P. syringae* pv tabaci did not cause disease symptoms in wild-type Col-0 nor in the *AtrbohD* mutant indicating that *AtRBOHD* is not involved in nonhost resistance. The single mutants *Atgox1* and *Atgox3* showed enhanced disease susceptibility to the nonhost pathogen exhibiting significant chlorosis as previously shown.[10] Similar to the results of silencing *AtRBOHD* in the *Atgox1* mutant background, the double mutant *AtrbohD* x *Atgox1* has symptoms similar to that of single *Atgox1* mutant. Interestingly, the double mutant *AtrbohD* x *Atgox3* did not have any disease symptoms. Inoculation with the host pathogen *P. syringae* pv maculicola showed dramatic disease symptoms in all genotypes without any significant difference among them (**Fig. 1**).

In order to quantify the effects of *AtrbohD* x *Atgox* double mutants on bacterial growth, we syringe-inoculated the plants with two nonhost pathogens, *P. syringae* pv tabaci and *P. syringae* pv syringae B728A, and a host pathogen, *P. syringae* pv maculicola (**Fig. 2**). As reported previously, single mutants of *Atgox1*and *Atgox3* inoculated with nonhost pathogens (**Fig. 2A, B, D and E**) showed ~10-fold increase in bacterial population at 3 dpi when compared with the wild type, while the growth of the host pathogen *P. syringae* pv maculicola was not different in these mutants in comparison with the wild type.[10] In contrast to the *Atgox1* and *Atgox3* mutants, the *AtrbohD* mutant did not support a significant increase in bacterial growth after inoculation with the nonhost pathogens similar to what was observed in wild type (**Fig. 2A and B**). These results are in line with previous studies on the *AtrbohD* mutant inoculated with the avirulent pathogen *P. syringae* pv tomato DC3000 (*AvrRpm1*).[8] In the double mutant, *AtrbohD* x *Atgox1,* both nonhost pathogens tested grew ~10-fold more than the wild type (**Fig. 2A and B**) and was in agreement with the symptoms observed (**Fig. 1**). This finding indicates that *AtrbohD* x *Atgox1* double mutant behaved similar to *Atgox1* single mutant, in response to nonhost pathogen inoculation, and therefore we determined that *AtGOX1* functions independently of *AtRBOHD*.

Interestingly, the growth of nonhost pathogens in the double mutant *AtrbohD* x *Atgox3* was slightly compromised when compared with *Atgox3* single mutant (**Fig. 2D and E**). This result suggests that the *AtrbohD* mutation is epistatic to the *Atgox3* mutation and the former causes a reversion of the phenotypes observed in *Atgox3* upon inoculation with nonhost pathogens. The mechanism behind this reversion is unknown but perhaps is

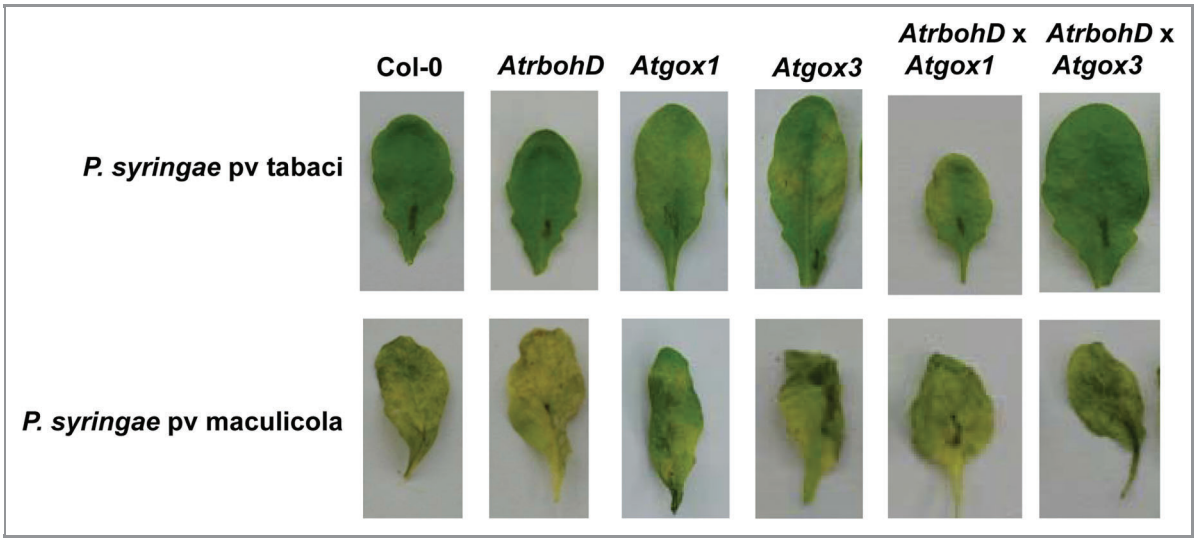

Figure 1. Effect of *AtrbohD* mutation in *Atgox1* and *Atgox3* mutants on symptom development associated with the nonhost pathogen *P. syringae* pv tabaci. Wild-type Col-0, single mutants *AtrbohD, Atgox1, Atgox3* and double mutants *AtrbohD* x *Atgox1* and *AtrbohD x Atgox3* were inoculated with the nonhost pathogen *P. syringae* pv tabaci and *P. syringae* pv maculicola at 1 × 10^4 CFU/ml. Symptoms were evaluated at 4 d post-inoculation.

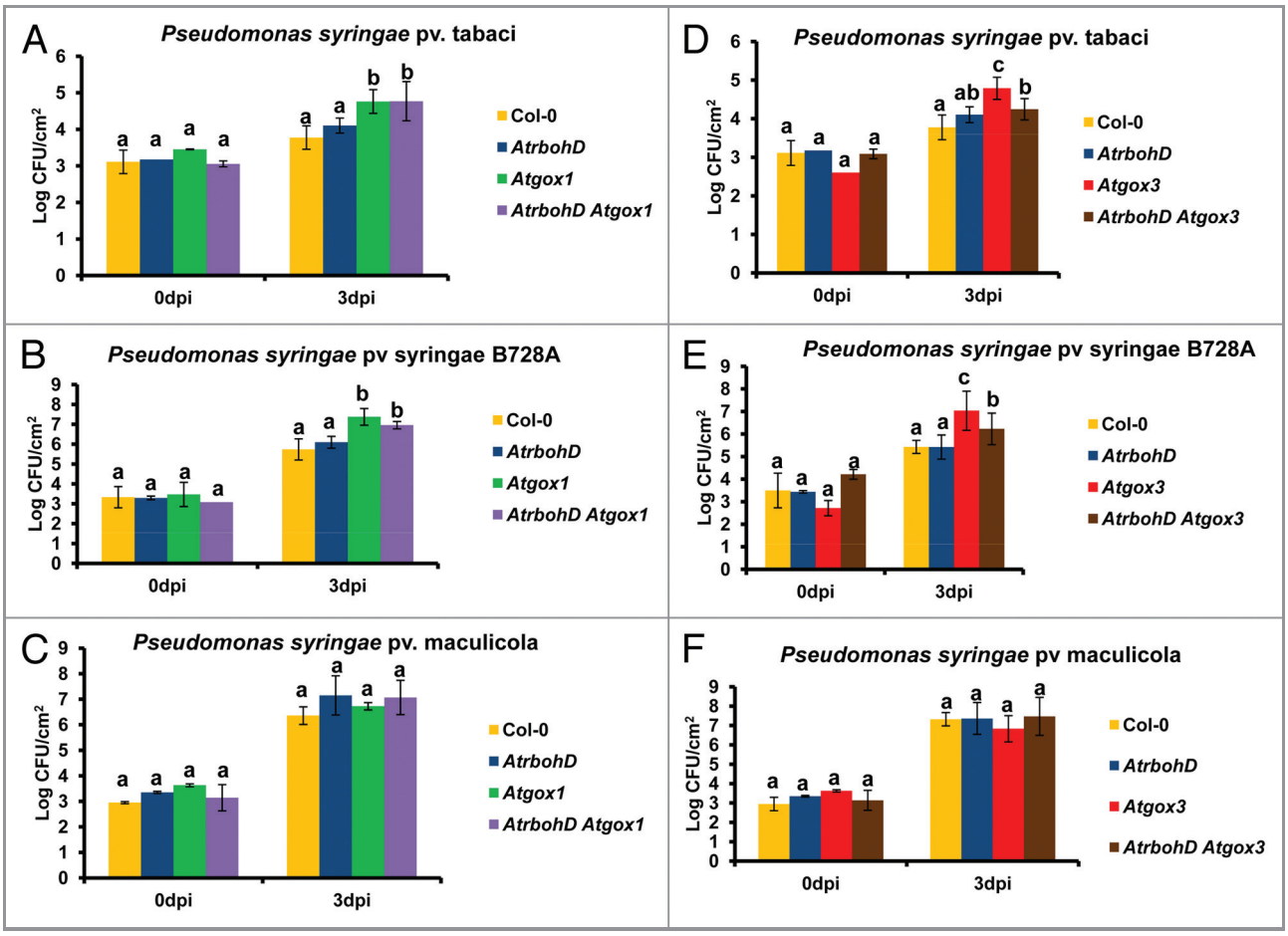

Figure 2. Differential response of *AtrbohD* x *Atgox1* and *AtrbohD* x *Atgox3* after inoculation with the nonhost pathogens *P. syringae* pv tabaci and *P. syringae* pv syringae B728A. Wild-type Col-0, single mutants *AtrbohD, Atgox1, Atgox3* and double mutants *AtrbohD* x *Atgox1* and *AtrbohD* x *Atgox3* were inoculated with the nonhost pathogen *P. syringae* pv tabaci (A and D), *P. syringae* pv syringae (B and E) and *P. syringae* pv maculicola (C and F) at 1×10^4 CFU/ml. At 0 and 3 dpi, two leaf samples (0.5 cm^2) from four biological replicates were collected, grounded, serially diluted and plated. Bacterial colonies were counted after two days. Bars represent means and standard deviation. One way ANOVA was used to determine statistical significance among treatments. After significance was found, LSD (least significant difference) test was used to determine differences between genotypes. Means with the same letter for a given time point are not significantly different at $p < 0.05$.

related to the accumulation of the phytoalexin camalexin as observed in the *cat2 AtrbohD* double mutant or involves accumulation of defense metabolites by the *AtrbohD* mutant.[8] The discrepancy between these and our previous results[10] wherein silencing of *AtRBOHD* in *Atgox1* and *Atgox3* mutant did not show any effect on disease symptoms and the apparent additive effect in the bacterial growth is probably due to the cross-silencing of *AtRBOHF*. None of the mutants tested were hyper-susceptible to the host pathogen *P. syringae* pv maculicola (**Fig. 2C and F**), indicating that neither *AtGOX* genes nor *AtRBOHD* are involved in basal resistance responses.

In conclusion, we show that there is no additive effect of disease symptoms or bacterial growth in the *AtrbohD* x *Atgox1* and *AtrbohD* x *Atgox3* double mutants when compared with individual mutants. Slight additive effect observed previously[10] upon silencing of *AtRBOHD* in *Atgox1* and *Atgox3* mutants was most likely due to cross-silencing of *AtRBOHF*. These results further prove that GOX plays a role in nonhost resistance independent of NADPH oxidase.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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