Engineered Polyamine Catabolism Preinduces Tolerance of Tobacco to Bacteria and Oomycetes

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Polyamine oxidase (PAO) catalyzes the oxidative catabolism of spermidine and spermine, generating hydrogen peroxide. In wild-type tobacco (Nicotiana tabacum 'Xanthi') plants, infection by the compatible pathogen Pseudomonas syringae pv tabaci resulted in increased PAO gene and corresponding PAO enzyme activities; polyamine homeostasis was maintained by induction of the arginine decarboxylase pathway and spermine was excreted into the apoplast, where it was oxidized by the enhanced apoplastic PAO, resulting in higher hydrogen peroxide accumulation. Moreover, plants overexpressing PAO showed preinduced disease tolerance against the biotrophic bacterium P. syringae pv tabaci and the hemibiotrophic oomycete Phytophthora parasitica var nicotianae but not against the Cucumber mosaic virus. Furthermore, in transgenic PAO-overexpressing plants, systemic acquired resistance marker genes as well as a pronounced increase in the cell wall-based defense were found before inoculation. These results reveal that PAO is a nodal point in a specific apoplast-localized plant-pathogen interaction, which also signals parallel defense responses, thus preventing pathogen colonization. This strategy presents a novel approach for producing transgenic plants resistant to a broad spectrum of plant pathogens.

The resistance of plants to invading pathogens is mediated by a complex array of defense responses (Goodman and Novacky, 1994; Jackson and Taylor, 1996) and nonspecific immunity to subsequent infection known as systemic acquired resistance (SAR; Ryals et al., 1996). The generation of reactive oxygen species (ROS), the oxidative burst, in response to microbial pathogen attack is a ubiquitous early part of the resistance mechanisms of plant cells in both compatible and incompatible plant-pathogen interactions. Several studies of plant-pathogen interactions and those modeled by elicitor treatment of cultured cells have suggested that although ROS generation by NADPH oxidase is a dominant mechanism in most plant species, more mechanisms could operate; in French bean (Phaseolus vulgaris) cells treated with a cell wall elicitor from Colletotrichum lindemuthianum, the major source of ROS was dependent on an extracellular peroxidase (Bolwell et al., 2002). The second component, the extracellular alkalization, occurs as a result of the Ca\(^{2+}\) and proton influxes and the K\(^{+}\) efflux common to most elicitation systems as one of the earliest responses. The third component, the actual reductant/substrate, has remained elusive (Bolwell et al., 2002).

Polyamines (PAs) are aliphatic amines that in plants derive either from Arg or Orn, via the Arg decarboxylase (YADC; EC4.1.1.19) or the Orn decarboxylase (ODC; EC4.1.1.17) pathway, respectively. Common PAs include diamine, putrescine (Put), and higher PAs (spermidine [Spd] and spermine [Spm]). Put, the precursor of higher PAs, is produced by the ADC and/or ODC pathway, and Spd and Spm biosynthesis requires the concerted action of spermidine synthase (SPDS; EC 2.5.1.16)/S-adenosyl-l-Met decarboxylase (SAMDC; EC 4.1.4.50) and spermine synthase (SPMS; EC 2.5.1.22)/SAMDC, respectively. That PAs are linked to protection against stress challenges in plants has long been proposed, based mostly on studies with pharmacological means and alterations of the intracellular PA titers (Alcazar et al., 2006).

The best-known enzyme that catabolizes higher PAs, generating hydrogen peroxide (H\(_2\)O\(_2\)) and reducing intracellular PA titers, is polyamine oxidase (PAO; EC 1.5.3.3). PAOs are localized to peroxisomes and in the apoplast (Rea et al., 2004; Moschou et al., 2008d). Recently, we showed that during abiotic stress, apoplastic PAO is responsible for apoplastic ROS generation (Moschou et al., 2008a, 2008b). Upon abiotic stress, Spd is secreted into the apoplast, where it is oxidized by PAO, producing H\(_2\)O\(_2\). Depending on its
levels ("signatures") and the intracellular PA homeostasis, the generated H$_2$O$_2$ signals tolerance-effector genes to abiotic stress or induces the execution of the programmed cell death (PCD) syndrome (Moschou et al., 2008a).

Yoda et al. (2003, 2006) reported that in tobacco (Nicotiana tabacum) plants resistant to Tobacco mosaic virus (TMV), PAO expression and PA titers increased in tissues exhibiting the TMV-induced hypersensitive response (HR), an incompatible plant-pathogen interaction. Cell death caused by TMV infection or cryptogein, an oomycete-originated elicitor, was partially mediated by H$_2$O$_2$ generated through PA catabolism. The substrate of PAO for H$_2$O$_2$ production was Spd, which accumulated in the apoplast during HR elicitation. Thus, PAO RNA interference lines exhibited significantly reduced HR rates when treated with cryptogein (Yoda et al., 2006). Moreover, Takahashi et al. (2003) provided evidence for the correlation of Spm oxidation and induction of HR-associated and defense-related genes. In addition, tolerance to TMV infection by treatment with Spm was modulated independently of salicylate (SA; Takahashi et al., 2003).

The previous studies were focused on the putative role of PAO in incompatible plant-microbe interactions. To further our understanding of the potential role of PA catabolism in plant-pathogen interactions, we examined whether or not the apoplastic PAO, a H$_2$O$_2$-producing enzyme, participates in the defense responses against three compatible pathogenic models using tobacco cv Xanthi transgenic plants overexpressing (S-PAO) and down-regulating (A-PAO) the maize (Zea mays) PAO gene (Moschou et al., 2008b). These plants were challenged with (1) the biotrophic bacterium Pseudomonas syringae pv tabaci (PS), (2) the hemibiotrophic oomycete Phytophthora parasitica var nicotianae (PP), and (3) the RNA virus Cucumber mosaic virus (CMV). PS causes "wildfire," one of the most destructive diseases affecting field tobacco plants. Oomycetes are fungus-like eukaryotic microorganisms that are aggressive pathogens of plants and animals. The genus Phytophthora consists of over 60 different species; all but three species are plant pathogens and are responsible for the most serious diseases of dicotyledonous plants, including potato late blight and sudden oak death (Erwin and Ribeiro, 1996; Appiah et al., 2004). Oomycetes and true fungi employ similar infection strategies but are different in their cell wall modifications of the ADC protein, acting in concert with its de novo synthesis to efficiently maintain PA homeostasis. On the contrary, ODC specific activity was higher at 48 hpi compared with 12 hpi, when ADC protein content was higher, suggesting posttranslational modifications of the ADC protein, acting in concert with its de novo synthesis to efficiently maintain PA homeostasis. On the contrary, ODC specific activity was higher at 48 hpi compared with 12 hpi, when ADC protein content was higher, suggesting posttranslational modifications of the ADC protein, acting in concert with its de novo synthesis to efficiently maintain PA homeostasis. On the contrary, ODC specific activity was higher at 48 hpi compared with 12 hpi, when ADC protein content was higher, suggesting posttranslational modifications of the ADC protein, acting in concert with its de novo synthesis to efficiently maintain PA homeostasis. On the contrary, ODC specific activity was higher at 48 hpi compared with 12 hpi, when ADC protein content was higher, suggesting posttranslational modifications of the ADC protein, acting in concert with its de novo synthesis to efficiently maintain PA homeostasis.

RESULTS

PA Metabolism Is Altered by Compatible Plant-Pathogen Infections

In wild-type tobacco leaves infiltrated with the pathogenic bacterium PS strain SFP-2124, PAO was an early-responsive gene; the abundance of its transcript level increased significantly at 12 h postinoculation (hpi) compared with the corresponding control (mock treatment), as shown by reverse transcription (RT)-PCR analysis (Fig. 1A; NtPAO gene). PAO protein and the specific PAO enzymatic activities showed similar increases, thus showing maximum levels at 12 hpi (Fig. 1B; P < 0.05). PAO specific activity increased 3.5-fold compared with the corresponding mock-treated plants at 12 hpi, and at 24 and 48 hpi PAO activity returned to basal levels (Fig. 1C; P < 0.05). Thus, PAO showed a transient early increase during pathogenic attack.

To examine whether increased PA oxidation induced the PA biosynthetic pathway in an effort to maintain intracellular PA homeostasis, the main PA biosynthetic enzymes ADC, ODC, SAMDC, SPDS, and SPMS were monitored. In fact, ADC specific activity was significantly induced upon treatment with the pathogen (58% at 12 hpi, 3-fold at 24 and 48 hpi; P < 0.05; Fig. 2, A and B). ADC protein levels increased as well at all time points examined, mostly at 12 hpi (Fig. 2B), suggesting de novo ADC protein synthesis (Fig. 1B; P < 0.05). Interestingly, ADC specific activity was higher at 48 hpi compared with 12 hpi, when ADC protein content was higher, suggesting posttranslational modifications of the ADC protein, acting in concert with its de novo synthesis to efficiently maintain PA homeostasis. On the contrary, ODC specific activity showed a minor initial increase (28% at 12 hpi) and decreased progressively thereafter (10% and 86% at 24 and 48 hpi, respectively; Fig. 2A, ODC). SAMDC specific activity followed a similar trend to ODC, with an initial increase (approximately 25% at 12 hpi) and a reduction thereafter (90% at 24 and 48 hpi; Fig. 2A; P < 0.05). Interestingly, SPDS specific activity exhibited a significant initial increase (2.5-fold at 12 hpi) and decreased thereafter (50% and 83% at 24 and 48 hpi; Fig. 2A; P < 0.05). SPMS could not be detected in that

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developmental stage. Thus, ADC seems to be the main route for PA titer maintenance in compatible interactions, while ODC possesses this role in incompatible ones (Negrel et al., 1984; Yoda et al., 2003, 2006).

It was of interest to examine the alterations in intracellular and intercellular PAs in wild-type tobacco brought about by the pathogen infections. All three main PAs, Put, Spd, and mostly Spm, significantly increased intracellularly at 12 hpi (Fig. 2C). Spm and Put exhibited 2.5- and 2-fold increases, respectively, whereas Spd increased only slightly (Fig. 2C; \( P < 0.05 \)). On the other hand, all three PAs decreased at 24 and 48 hpi (Fig. 2C). Interestingly, the faster decline was that of Spm (approximately 50% at 24 and 48 hpi; Fig. 2C), while Put and Spd declined at a lower rate (Fig. 2C). On the contrary, in the apoplastic compartment, the most striking increase was that of Spm (18-fold increase at 12 hpi); Put did not accumulate significantly, and Spd showed only a minor increase (Fig. 2D).

**Figure 1.** Abundance of *NtPAO* mRNA levels, PAO protein, and specific activity in wild-type plants treated with PS. A, RT-PCR analysis for the *NtPAO* mRNA expression level at 12 hpi. Samples were standardized using the *NtUbi* gene in wild-type plants treated with PS, and PCR products were electrophoretically resolved on ethidium bromide (0.5 \( \mu \text{g mL}^{-1} \))-containing agarose gels (1.5%, w/v). B, Western-blot analysis of the PAO protein levels using an anti-M-PAO specific antibody (50 \( \mu \text{g lane}^{-1} \)) preinoculation and at 12, 24, and 48 hpi. *, Mock treatment; +, PS inoculation. C, PAO specific activity levels in relative activity units (RU). Data are means \( \pm \text{SE} \) of three independent experiments, and asterisks indicate statistical significance from the corresponding controls at \( P < 0.05 \).

Compatible Plant-Pathogen Interaction Results in Increased Apoplastic \( \text{H}_2\text{O}_2 \)

We showed recently that abiotic stress induces secretion of Spd into the apoplastic compartment, where it is oxidized by the cell wall-associated PAO; the generated \( \text{H}_2\text{O}_2 \), depending on its size, signals either molecular stress defense responses or PCD (Moschou et al., 2008c). In this work, we followed the same strategy in order to investigate whether a similar phenomenon takes place in the compatible plant-pathogen interactions. Upon inoculation with PS bacteria, in S-PAO plants a 3.8-fold increase in the apoplastic \( \text{H}_2\text{O}_2 \) levels at 12 hpi was apparent and declined thereafter, as indicated using the \( \text{H}_2\text{O}_2 \)-specific dye 3,3'-diaminobenzidine (Fig. 3, A and B), while in A-PAO and wild-type plants a slight increase of \( \text{H}_2\text{O}_2 \) levels was found, which remained somewhat higher than in the corresponding mock-treated plants (Fig. 3B; \( P < 0.05 \)). To further verify the localization of \( \text{H}_2\text{O}_2 \), transmission electron microscopy (TEM) specific for \( \text{H}_2\text{O}_2 \) was used, and the results confirmed its extracellular increase, as evidenced by the electron-dense black spots detected specifically in the apoplast (Fig. 4).

Also, PS-elicited \( \text{H}_2\text{O}_2 \) accumulation was evident soon after inoculation in the apoplast of the S-PAO transgenic plants, while only a moderate increase was found in A-PAO and wild-type plants (Fig. 4). These results support a direct positive correlation between \( \text{H}_2\text{O}_2 \) accumulation and PAO activity in the apoplast of PS-infected plants and that PAO directly affects the apoplastic ROS load upon elicitation.

**S-PAO Transgenic Tobacco Exhibits Tolerance to Virulent Bacterial Pathogens and to a Hemibiotrophic Oomycete But Not to a Virus**

To evaluate whether alteration of the PAO gene affects plant disease resistance, S-PAO and A-PAO plants were assessed (Moschou et al., 2008a, 2008b). The S-PAO plants showed a more than 10-fold increase, whereas the A-PAO plants showed a 50% decrease, of PAO specific activity when compared with wild-type plants (Moschou et al., 2008b). The transgenic tobacco plants were challenged with two distinct strains of the virulent pathogen PS, with the hemibiotrophic oomycete PP, and with CMV. One PS strain with mild pathogenicity aggressiveness, PS BPIC1514, and a second strain with high pathogenicity aggressiveness, PS SFP-2124, were initially used. The latter was chosen because it induces more rapidly the wildfire symptoms. No symptoms were observed after inoculation with the most virulent PS in S-PAO plants, in contrast to A-PAO and wild-type plants, which...
developed typical symptoms of infection by both PS and PP pathogens (Fig. 4A). Symptoms in wild-type and A-PAO plants appeared as spatial chlorophyll loss leading to tissue collapse (Fig. 4A). On the contrary, no phenotypical differences were observed between the genotypes used when challenged with CMV (Fig. 4A).

To investigate if the lack of symptoms in the S-PAO plants was due to a lower growth rate of bacteria and lower growth of PP mycelia, population analyses for PS and estimation of PP radial growth were performed, respectively (Fig. 4, B and C). The growth rate of PS and endophytic growth of PP in S-PAO plants were significantly lower compared with the corresponding values in A-PAO and wild-type plants (Fig. 4, B and C; \( P < 0.05 \)). The same trend was observed when using the mild PS strain (data not shown). More specifically, although as in wild-type plants the bacterial population in S-PAO plants increased significantly at 24 hpi, the rate of PS growth was significantly lower (Fig. 4B; \( P < 0.05 \)). Thus, in wild-type plants and A-PAO plants as well, the increase in growth rate of PS was dramatic, reaching 10-fold higher colony-forming units compared with S-PAO plants (Fig. 4B; \( P < 0.05 \)). The PS growth rate in A-PAO plants was slightly higher at 24 hpi (Fig. 4B; \( P < 0.05 \)). During the PP infection of tobacco leaves, S-PAO plants were dramatically more tolerant to PP infection (Fig. 4C; \( P < 0.05 \)). Interestingly, the endophytic growth in A-PAO leaves was slightly slower compared with that of control leaves at 8 d postinoculation, but eventually the whole leaf was infected in a manner similar to PP infection of wild-type tobacco.

**Figure 2.** Specific activity of ADC, ODC, SAMDC, SPDS, and PA titers in wild-type plants treated with PS. A, ADC, ODC, SAMDC, and SPDS specific activities preinoculation and at 12, 24, and 48 hpi in relative units (RU). B, Western-blot analysis of the ADC protein levels using an anti-ADC specific antibody (30 \( \mu \)g lane\(^{-1}\)) preinoculation and at 12, 24, and 48 hpi. –, Mock treatment; +, PS inoculation. C, Put, Spd, and Spm titers preinoculation and at 12, 24, and 48 hpi in relative units compared with the corresponding mock-treated plants. D, Apoplastic Put, Spd, and Spm titers at 12 hpi in relative units compared with the corresponding mock-treated plants. Data are means ± se of three independent experiments, and asterisks indicate statistically significant differences from the corresponding controls at \( P < 0.05 \).
leaves (Fig. 4C; P < 0.05). Finally, no differences in the multiplication rates were found in the examined genotypes challenged with CMV (Fig. 4D).

**Modulation of PAO Induces Primary and Secondary Biotic Stress Responses Independent of SA**

Primary defense responses of plants to pathogens involve cell wall-associated modifications of cellulose and hemicellulose-interacting compounds, such as pectins, lignins, and callose. This prompted us to examine if the altered PAO levels resulted in differences in the structures of plant cell walls preinoculation and postinoculation with PS in the wild-type and PAO transgenic plants. Pectin content increased both preinoculation and postinoculation in S-PAO plants, while an increase in the pectin content was evident only postinoculation in A-PAO and wild-type plants (Fig. 5A). Lignin content was only slightly modulated in S-PAO plants, while it was reduced in A-PAO plants when compared with wild-type plants (10%; Fig. 5, B and C). Moreover, callose deposition was significantly higher in S-PAO in contrast to A-PAO plants (Fig. 5D; P < 0.05). These data reinforce the view that increased PAO activity in the apoplast influences the primary defense responses against pathogens.

On the other hand, secondary plant defense responses involve readjustment of the expression of a wide array of defense-related genes. Among the best-known examples are the genes encoding for the pathogenesis-related (PR) proteins, while genes such as PrxC1 and PrxN1 have been shown to participate in the Spm signaling pathway (Yamakawa et al., 1998). One would expect that the increased PA oxidation in S-PAO plants and their higher tolerance could imply differential induction of the respective defense-related genes, since their induction has been shown to be signaled by, among others, H$_2$O$_2$, especially in the case of SIPK (Kroj et al., 2003). The PR-1a and PR-5db mRNA levels were significantly higher in mock-treated S-PAO plants, as shown by RT-PCR, in contrast to A-PAO and wild-type plants (Fig. 6). On the other hand, these genes were down-regulated postinoculation in S-PAO plants and were slightly induced in A-PAO and wild-type plants (Fig. 6, PR-1a and PR-5db). Prx-C1 transcript levels were not significantly different between the genotypes examined (Fig. 6). Also, Prx-N1 was significantly higher in mock-treated S-PAO plants, while in the inoculated S-PAO, A-PAO, and wild-type plants, Prx-N1 was not detectable (Fig. 6). In addition, SIPK and WIPK followed the same trend as PR-1a, PR-5db, and Prx-N1. On the other hand, NtPAO under these conditions was detectable only in A-PAO plants postinoculation. It should be noted that the increase of the NtPAO transcript was expected, since in these transgenic lines the reduction of PAO activity is due to the translational inhibition and not to the posttranscriptional gene silencing that would not allow an increase in the transcript, as suggested previously (Moschou et al., 2008b).

To further support that differential oxidation of PAs had affected the observed transcript accumulation...
postinoculation, we supplied PAs exogenously that were infiltrated in the apoplast and followed the expression levels of \textit{PR-1a} and \textit{PR-5db} at 12 h after treatment. Exogenous supply of PAs in wild-type plants efficiently induced \textit{PR-1a} and \textit{PR-5db} in a dose-responsive manner. In contrast, in S-\textit{PAO} plants, \textit{PR-1a} transcript did not increase; instead, both transcripts declined, except with 10 mM Spd, when an increase of \textit{PR-5db} was observed, suggesting the activation of a different pathway (Fig. 7). On the other hand, addition of PAs to the A-\textit{PAO} plants led to \textit{PR-1a} and \textit{PR-5db} mRNA accumulation, at a slower rate since only high levels of Spd and Spm (10 mM) were effective in these plants (Fig. 7). Put exerted a similar effect in A-\textit{PAO} and wild-type plants (Fig. 7), whereas in S-\textit{PAO} plants, a significant reduction of both transcripts was found. These results further confirm that PA oxidation in the apoplast efficiently induces PR-encoding genes, while in S-\textit{PAO} plants they were induced prior to infection. Thus, controlled PA oxidation is responsible for the induction of a wide array of defense genes.

Genes such as \textit{SIPK} and \textit{WIPK} are induced by increased SA (Sharma et al., 2003). To reveal whether SA participates in the tolerance observed in S-\textit{PAO} plants and, more specifically, in \textit{SIPK} and \textit{WIPK} induction, total SA titers were determined in tobacco leaves preinoculation and postinoculation. Postinoculation with PS, S-\textit{PAO} plants exhibited significantly higher levels in contrast to A-\textit{PAO} plants, when compared with wild-type plants, while SA titers progressively declined at 24 and 48 hpi, although again, S-\textit{PAO} plants showed significantly higher SA titers (Fig. 8; \( P < 0.05 \)). On the other hand, in control plants, the differences in total SA titers were not significantly different (Fig. 8, inset). These results reveal that the observed induction of \textit{SIPK} and \textit{WIPK} (and the rest of the SAR-associated genes) in S-\textit{PAO} plants is not SA dependent.
DISCUSSION

Plant pathogens employ a wide array of offensive strategies, and plants activate a similar array of defense responses to thwart pathogen attack (Hirt, 2002). However, the constitutive overproduction of H$_2$O$_2$ could be detrimental to plant cells inducing the PCD syndrome. To overcome this, we employed a strategy that involves the spatial partitioning of an apoplastic H$_2$O$_2$-generating enzyme (PAO) with its substrate (Spd/Spm), which only under stress conditions meet to produce H$_2$O$_2$. Significant H$_2$O$_2$ production in the apoplast is facilitated by the absence of antioxidant machinery in that compartment. Moreover, the absence of H$_2$O$_2$-sensitive partners in the apoplast grants this compartment the unique ability to withstand high ROS load. Recently, apoplastic PAO was identified as one enzyme mediating the generation of H$_2$O$_2$ in the apoplast via oxidation of higher PAs under abiotic stress (Moschou et al., 2008b). Upon abiotic stress, Spd is secreted into the apoplast, where it is oxidized by PAO, and the resulting H$_2$O$_2$, depending on its “signature,” signals either expression of defense gene(s) conferring tolerance or of genes inducing PCD syndrome. Thus, overexpression of PAO in tobacco plants resulted in high apoplastic H$_2$O$_2$ content and increased sensitivity to abiotic stress (Moschou et al., 2008a, 2008b).

In this work, we attempted to establish the role of PAO in biotic stress following leaf infection with bacterium, oomycete, and viral pathogens, using transgenic tobacco plants with overexpressed or down-regulated PAO gene. Postinfection with PS, the

Figure 5. In situ pectin, lignin, and callose quantification in wild-type (WT) and S-PAO and A-PAO transgenic lines, and SA quantitation analysis. A, Pectin-specific TEM results. Plants were mock-inoculated or inoculated with PS at 24 hpi. Ap, Apoplast; Cl, chloroplast; Vc, vacuole. B, In situ lignin content (thicker veins and cell walls shown in gray). C, Lignin content (per mg dry weight [DW]). D, In situ detection of callose deposition in mock- or PS-inoculated leaves at 24 hpi (blue-white areas surrounding the cells). Arrows indicate the accumulation of pectin, lignin, and callose. [See online article for color version of this figure.]

Figure 6. Abundance of mRNA of PR-1a, PR-5db, PrxC1, PrxN1, SIPK, WIPK, and NtPAO (endogenous) genes before and after inoculation with PS in wild-type (WT) and S-PAO and A-PAO transgenic plants. mRNA levels were estimated using semiquantitative RT-PCR, and as a loading control the Ubi gene was used. PCR products were electrophoretically resolved on ethidium bromide (0.5 µg mL$^{-1}$)-containing agarose gels (1.5%, w/v). --, Mock treatment; +, PS inoculation.
NtPAO gene was induced and PAO immunoreactive protein accumulated shortly after in wild-type tobacco plants (Fig. 1). Concomitantly, the PA biosynthetic enzymes were induced with ADC to be the most responsive enzyme to maintain PA homeostasis during compatible plant-pathogen interactions and to supplement PAO with substrates (Fig. 2). On the contrary, ODC was shown to be the responsive enzyme for PA synthesis in tobacco plants during the HR response (Yoda et al., 2003, 2006). The maximum increase in the PA biosynthetic activities was mirrored by a parallel increase of the corresponding PA titers at 12 hpi, mostly those of Spm in the apoplastic compartment, which could serve as substrate for the PAO-derived H2O2 production in the apoplast (Fig. 3).

The increase of H2O2 was significantly more evident in S-PAO plants. Thus, PAO actively participates in the apoplastic ROS production. Moreover, S-PAO plants exhibited tolerance to PP, mostly to the latter. On the contrary, A-PAO plants showed slightly increased symptomatology (Fig. 4) with respect to the corresponding wild-type plants infected by PS and also an increased colonization rate. A slight delay in the PP mycelial growth in A-PAO plants could be attributed to the elevated levels of Spm in the apoplast, which down-regulate Put and Spd synthesis of the oomycetes (Chibucos and Morris, 2006); Phytophthora species have been shown to express transporters for such PAs, involved in their direct uptake as well as in their regulation in the mycelial cells during vegetative growth (Chibucos and Morris, 2006). That CMV transmission was not affected in the examined transgenic genotypes could be due to the fact that PAO could not affect the intracellular multiplication of the virus. Thus, increased higher PA oxidation results in increased tolerance to bacteria and oomycetes, whereas lower PA oxidation does not seem to be detrimental to the overall tolerance. The latter findings suggest that additional mechanisms could supplement the losses in the capacity of PA oxidation, such as NAD(P)H oxidase and peroxidases.

Moreover, we strived to examine if overexpression of PAO had resulted in the induction of a preinfection response, which could as well contribute to tolerance of S-PAO plants to both the bacterium and the oomycete. Candidates for that could be the activation of the host’s basal defense (i.e. alterations in the structure of the cell wall and/or expression of pathogenesis-related genes). The first line of defense against the pathogenic challenge is associated with the plant cell wall, in which certain modifications take place during attack, respecting the rule “the harder the better.” These modifications involve increased pectin and lignin contents and callose deposition, facilitating plant defense. Thus, S-PAO plants showed enhanced pectin content, under both normal growth and biotic stress conditions. Furthermore, lignin content was only slightly higher in S-PAO plants when compared with wild-type plants, whereas the opposite was true for A-PAO plants. In addition, callose depositions in S-PAO plants were significantly higher compared with wild-type and A-PAO plants (Fig. 5). The latter could render PP unable to infect tobacco plants, as plant defense

**Figure 7.** Abundance of PR-1a and PR-5db mRNAs after exogenous PA application in wild-type (WT) and S-PAO and A-PAO transgenic plants.

**Figure 8.** Accumulation of SA in PS-inoculated leaves at 0, 12, 24, and 48 hpi. The inset shows total SA titers in control plants. FW, Fresh weight; WT, wild type.
responses to oomycete involve callose and lignin deposition (Walters, 2003).

In our earlier work, we showed that in S-PAO plants antioxidant genes were preinduced (Moschou et al., 2008a), as in the case of SAR. Additionally, an acute exposure to O$_3$, which produces O$_2^-$ and H$_2$O$_2$ in the apoplast, elicited changes in the levels of plant hormones, such as ethylene, SA, and jasmonic acid, and activated several other signaling pathways leading to changes in gene expression (Ahlfors et al., 2004). The primary signaling events induced by apoplastic O$_2^-$-derived H$_2$O$_2$ involved mitogen-activated protein kinase (MAPK) activation. Plant MAPK cascades appear to be involved in a multitude of biotic and abiotic stress responses, hormone responses, and regulation of cell division, and overall they are expressed throughout the development of plants. In tobacco, O$_3$ increased the activity of SIPK and WIPK (Samuel and Ellis, 2002) oriented to the activation of the PR proteins (Kroj et al., 2003), while tobacco SIPK was described as a SA-activated MAPK (Zhang and Klessig, 1998).

In plant-pathogen interactions, MAPKs are involved in controlling subsets of genes activated during defense responses (Kroj et al., 2003). This prompted us to investigate the effect of the increased PA-derived apoplastic H$_2$O$_2$ production in the induction of MAPKs that regulate the expression of SAR-activated genes. Thus, in S-PAO plants, some important genes that contribute to tolerance were induced prior to inoculation. These genes included PR-1a, PR-5db, PrxN1, and two MAPKs, namely SIPK and WIPK. On the contrary, PrxC1 was not induced (Fig. 6). The PR proteins are considered to be involved in the postinfection defense against biotic stress challenge, while genes such as PrxC1 and PrxN1 have been shown previously to be involved in the Spm signaling pathway that takes place during TMV infection of tobacco plants and to depend upon Spm oxidation in the apoplast of tobacco plants for their activation (Takahashi et al., 2003). Recently, Spd oxidation was also shown to activate PR-1a transcription in tobacco plants (Lazzarato et al., 2009). Interestingly, these genes were not further induced in S-PAO plants postinoculation, whereas they were further induced in wild-type and A-PAO plants. The differential response in S-PAO plants was very similar to that reported previously, in which the onset of stress resulted in the inhibition of antioxidant gene induction (Negrel et al., 1984; Moschou et al., 2008a; Mitsuya et al., 2009). Moreover, by supplying exogenously higher PAs in the apoplast of S-PAO plants, instead of higher SAR-associated gene accumulation, as was the case in wild-type and A-PAO, a decline in the corresponding transcripts was observed (Fig. 7). Thus, the view that an additional PAO-dependent response is not significantly implicated in the biotic tolerance response of S-PAO plants is reinforced.

The preinduced increments observed in S-PAO plants could be dictated by the SA. Moreover, SA increase has been considered to be a nodal point in ROS production during pathogenic challenge, and SAR is preceded by an increase in the SA content, although SAR-associated gene induction was shown to be independent of SA accumulation in some cases (Yasuda et al., 2003). Thus, since genes like SIPK and WIPK were activated, one would expect that the increase of SA in S-PAO plants would be responsible for the induction of the defense genes and also for the concomitant cell wall modifications. Interestingly, under control conditions, no increase in SA content could be found in either S-PAO or A-PAO plants, suggesting that the induction of these genes was SA independent (Fig. 8). Moreover, the higher SA content postinoculation in the S-PAO plants was probably due to lower postinoculation PS levels in the leaves of these plants, since bacteria are at least partly responsible for the inhibition of the defense responses, like SA accumulation (Vivian and Arnold, 2000; Abramovitch et al., 2006). These data reinforce the view that increased PA oxidation can solely activate at least some defense responses independently of SA.

In summary, this work provides evidence that the apoplastic PAO is an important player in defense signaling during compatible plant-pathogen interactions, excluding compatible viral infections. Moreover, PAO induces plant tolerance mechanisms that can lead to tolerance against devastating plant diseases. Thus, genetic engineering for PAO overexpression can be an efficient method for enhancing the tolerance of plants to bacteria and oomycetes.

**MATERIALS AND METHODS**

**Plant Material and Grown Conditions**

Tobacco plants (*Nicotiana tabacum* 'Xanthi') were grown in a growth chamber with irradiance of approximately 100 µmol m$^{-2}$ s$^{-1}$, temperature of 25°C ± 2°C, 16/8-h photoperiod, and 75% relative humidity. Transgenic tobacco S-PAO (lines S2.2 and S4) and A-PAO (lines A2 and A6) plants were constructed as described previously (Moschou et al., 2008b). Data presented from transgenic plants derived from lines S2.2 and A2, whereas lines S4 and A6 showed similar results.

**PA Analysis, Protein Extraction, and Enzyme Assays**

Apoplastic and total PAs were determined as described previously (Kotzabasis et al., 1993; Moschou et al., 2008b). Total proteins were extracted as described (Primikirios and Roubelakis-Angelakis, 2001). For the PAO assay, a spectrophotometric method developed by Federiko et al. (1985) was used with minor modifications. A radiometric method was also used for PAO assays according to Paschalidis and Roubelakis-Angelakis (2005a). ADC, ODC, and SAMDC were assayed by measuring the release of $^{14}$CO$_2$; L-[$^{1-14}$C]Arg, L-[$^{1-14}$C]Orn, and L-arginine-1-$^{13}$C Met, respectively (American Radiolabeled Chemicals), were used as radioactive substrates. Labeled CO$_2$ was counted in an LS 6000SE (Beckman) scintillation counter (Paschalidis and Roubelakis-Angelakis, 2005b). SDPS and SPMS were assayed by measuring the formation of Spd and Spm, respectively, according to Paschalidis and Roubelakis-Angelakis (2005a). Specific enzymatic activities were expressed as relative units with respect to the corresponding controls.

**Protein Gel Blotting, RNA Extraction, and DNA and RNA Gel Blotting**

Total protein extracts were electrophoretically resolved, transferred to membranes, and hybridized against an anti-PAO maize (Zea mays) polyclonal...
antibody (Paschalidis and Roubelakis-Angelakis, 2005a) or an anti-ADC grape (Vitis vinifera) polyclonal antibody (Paschalidis and Roubelakis-Angelakis, 2005a). For RNA gel blotting, total RNA was extracted according to Miller (1972), transferred to a membrane, and hybridized to the corresponding 32P-labeled probe prepared using the RadPrime DNA labeling kit as described by the manufacturer (Invitrogen). Probes were prepared as described previously (Paschalidis and Roubelakis-Angelakis, 2005a, 2005b). X-ray films (Kodak) and low exposure times were used for visualization.

cDNA Cloning and RT-PCR

All genes assessed in this study were cloned in a pGEM T Easy vector (Promega) and sequenced. The primers used for RT-PCR and the cloning are described in Supplemental Table S1.

For quantitation, total mRNA from leaves was extracted and treated with RNase-free DNase I for 45 min at 37°C (Iandolo et al., 2004). The samples were then subjected to RT-PCR using poly(T) as primer and the Super RT enzyme according to the manufacturer’s instructions (Takara). The samples were normalized according to the NtActin gene (this was also replicated with the NtUbi gene). More specifically, samples were used for 20, 30, 35, and 40 cycles of PCR (4 min at 94°C initial denaturation, 30 s at 94°C cycling denaturation, 52°C–55°C primer annealing, 45–60 s extension, and a 2-min final extension) and analyzed using agarose gel electrophoresis to estimate whether the reaction was still in the logarithmic phase.

Bacterial Strains and Cultivation, Leaf Inoculation, and Bacterial Population Counts

Cultures of rifampicin-resistant Pseudomonas syringae pv tabaci strains BP1/C176 and SEP-2124 were grown for 24 h at 28°C on Luria-Bertani (LB) medium at pH 7.0. LB medium was from the recipe of Miller (1972). Bacteria were suspended in 10 mM MgCl2, and the density was determined photometrically (optical density at 600 nm).

Fully expanded leaves from 5- to 6-week-old tobacco Xanthi plants were used for bacterial and oomycete inoculation. Four-week-old tobacco plants were used for virulence assays. Plants were grown under greenhouse conditions and transferred to the laboratory 1 d before inoculation.

For virulence assays, P5 was grown at 28°C for 4 h in liquid LB medium supplemented with the appropriate antibiotic (rifampicin, 80 μg mL−1). Bacterial cells were harvested by spin-down at 2,800 rpm for 10 min in 4°C, washed twice in cold 10 mM MgCl2, and finally resuspended to an optical density at 600 nm of 0.3 (approximately 5 × 108 colony-forming units mL−1) in sterile 10 mM MgCl2. Bacterial suspensions (5 × 107 colony-forming units mL−1) obtained by serial dilution were infiltrated using a blunt syringe into the intercellular spaces of leaves from all transgenic lines and wild-type tobacco (at the five- to 10-leaf stage, abaxial surface) following the methods of Tsiamis et al. (1994), and plants were maintained in the laboratory with illumination at room temperature (24°C).

Bacterial multiplication in tobacco leaves was monitored by cutting leaf discs from the inoculated sites with a 0.6-cm-diameter borer, homogenizing them in 10 mL MgCl2, and serially diluting the homogenate, which was then spread onto LB agar with appropriate antibiotic (rifampicin, 80 μg mL−1) to allow colony development at 28°C (Tsiamas et al., 2000). Colonies were counted after incubating the plates for 48 h at 28°C.

Transmission Tests of CMV

Mechanical transmission tests were carried out using leaves from CMV-infected tobacco. The leaves were ground in phosphate buffer, and inoculation was performed by application to the third and fourth leaves of 5- to 7-week-old plants. Seven and 12 d post inoculation, separate samples were taken from inoculated leaves of all plants and stored at −20°C. Plants were kept in the greenhouse for 40 d. All samples were tested for the presence of CMV by RNA gel blot analysis for the CMV coat protein.

TEM for H2O2 and Pectin Localization

H2O2 was also detected by the cerium chloride (CeCl3) method, as described (Bestwick et al., 1997). In brief, small pieces (2–5 mm2) of tissues from the central laminar region of tobacco third leaves were incubated in freshly prepared 50 mM MOPS buffer, pH 7.2, containing 5 mM CeCl3, for 1 h. Subsequently, the samples were fixed in a mixture of 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM cacodylate buffer (CB), pH 7.2, for 1 h. After washing in CB, samples were postfixed overnight in 1% osmium tetroxide (prepared in CB), dehydrated in a graded ethanol series, and embedded in London Resin White. Ultrathin sections were examined by TEM at 80 kV without poststaining. To confirm the specificity of CeCl3 staining, for H2O2, samples were incubated for 20 min in 50 mM MOPS, pH 7.2, containing either 1 mM sodium azide (to inhibit peroxidase) or 25 mg mL−1 bovine liver catalase (to decompose H2O2). They were then transferred to CeCl3 solution, incubated for 1 h, and processed for TEM as described above. Some samples were fixed without the CeCl3 treatment. After postfixation in OsO4, samples were processed for TEM as outlined above.

For pectin detection, sections from central laminar regions of tobacco third leaves were fixed for 4 h in a mixture of 0.05% (w/v) ruthenium red and 3% (v/v) glutaraldehyde in 50 mM CB, pH 7.4. After washing in the same buffer, the samples were postfixed in 1% (w/v) osmium tetroxide. After dehydration in a graded series of acetone, they were embedded in Spurr’s low-viscosity resin (Spurr, 1969). Ultrathin sections were stained with uranyl acetate.

Lignin Detection and Quantification

Leaves were excised from inoculated plants and cleared from pigments by immersion in three changes of absolute methanol over 3 d. Lignification of leaf cells was visualized by incubating cleared leaves overnight in 1 mL of 1% (w/v) phloroglucinol in 70% (v/v) ethanol. The leaf tissue was then mounted on glass slides with a few drops of concentrated HCl. After 5 min, excess HCl was drained from the slides and replaced with deionized water for light microscopy.

Accumulation of lignin was quantified by the thioglycolic acid reaction according to Dos Santos et al. (2004). Lignin content was expressed as mg lignin-thioglycolic acid complex g−1 leaf dry weight using molar absorptivity of 17.87 g L−1 cm−1.

Callose Deposition

To visualize callose deposition, leaves were cleared in 95% ethanol, stained with aniline blue, and examined for fluorescence as described with a UV microscope (Adam and Somerville, 1996).

SA Analysis

For total SA extraction, 200 mg of leaf tissue was ground with liquid N2 and SA was extracted once with 1 mL of 90% (v/v) methanol and once with 1 mL of 100% (v/v) methanol. The two extracts were combined and dried under nitrogen gas. The extract was then resuspended in 1 mL of 3% TCA. Twenty microliters of HCl was added, and samples were incubated in boiling water for 30 min. The SA was extracted in cyclohexanone/ethyl ether/isopropanol (50:50:1), and the extract was dried under nitrogen gas and resuspended in 200 μL of methanol. Fifty microliters of each sample was analyzed using an HP 1100 high-performance liquid chromatograph (Hewlett-Packard). The mobile phase consisted of methanol/water/acetic acid (54:45:1). An isocratic flow rate of 0.550 mL min−1 was used, and SA was detected at 311 nm after elution from a 4 × 250-mm C-18 reverse-phase column (Analytenschnick) using a controlled column temperature of 30°C. The SA retention time was approximately 10 min.
Image and Statistical Analyses

Image and pixel analyses and mRNA quantification were performed with ImageJ 1.37v (rsb.info.nih.gov/ij), and statistical analysis was performed with SPSS 14v (www.spss.com).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: NtPAO, AB200262; M-PAO, AJ002204; NtPrx-1a, X12485; NtPrx-5dh, AB121785; NtPrxC1, AB027752; NtPrxN1, AB027753; NtSiPK, NTU94192; NtWIPK, AB052964; NtActin, X63603; NTUHI, U66264.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Sequences of the primers used in this study.

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LITERATURE CITED

Abramovitch RB, Anderson JC, Martin GB (2006) Bacterial elicitation and evasion of plant innate immunity. Nat Rev Mol Cell Biol 7: 601–611

Adam L, Somerville SC (1996) Genetic characterization of five powdery mildew disease resistance loci in Arabidopsis thaliana. Plant J 9: 341–356

Ahlfors R, Macioszek V, Rudd J, Broschei M, Schlichting R, Scheel D, Kangasjarvi J (2004) Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in Arabidopsis thaliana during ozone exposure. Plant J 40: 512–522

Alcazar R, Marco E, Cuevas JC, Patron M, Ferrando A, Carrasco P, Tiburcio AF, Allatella T (2006) Involvement of polyamines in plant response to abiotic stress. Biotechnol Lett 28: 1867–1876

Appiah AA, Jennings P, Turner JA (2004) Phytophthora ramorum: one pathogen and many diseases, an emerging threat to forest ecosystems and ornamental plant life. Mycologist 18: 145–150

Badreddine I, Lafitte C, Heux L, Skandalis N, Spanou Z, Martinez Y, Esquerre-Tugaye MT, Bulone V, Dumas B, Bottin A (2008) Cell wall chitosaccharides are essential components and exposed patterns of the phytopathogenic oomycete Aphanomyces euteiches. Euokaryot Cell 7: 91–108

Bestwick CS, Brown IR, Bennett MH, Mansfield JW (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to Pseudomonas syringae pv phaseoliciola. Plant Cell 9: 209–221

Bolwell GP, Bindschleder LV, Blee KA, Butt VS, Davies DR, Gardner SL, Gerlich C, Minibaeva F (2002) The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J Exp Bot 53: 1367–1376

Chibucos MC, Morris PF (2006) Levels of polyamines and kinetic characterization of their uptake in the soybean pathogen Phytophthora sojae. Appl Environ Microbiol 72: 3350–3356

Dos Santos WD, Ferrarese MLL, Finger A, Teixeira ACN, Ferrarese-Filho O (2004) Lignification and related enzymes in Glycina max root growth-inhibition by ferulic acid. J Chem Ecol 30: 1203–1212

Erwin CE, Ribeiro OK (1996) Phytophthora Diseases Worldwide. APS Press, St. Paul

Federico R, Angelini R, Cesta A, Pin i C (1985) Determination of diamine oxidase in lentil seedlings by enzymic activity and immunoreactivity. Plant Physiol 79: 62–64

Goodman RN, Novacky AJ (1994) The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. APS Press, St. Paul

Hirt H (2002) A new blueprint for plant resistance pathway. Nat Biotechnol 20: 450–451

Iandolino AB, da Silva FG, Lim H, Choi H, Williams LE, Cook DR (2004) High-quality RNA, cDNA, and derived EST libraries from grapevine (Vitis vinifera L.). Plant Mol Biol Rep 22: 269–278

Jackson AO, Taylor CB (1996) Plant-microbe interactions: life and death at the interface. Plant Cell 8: 1651–1668

Klement Z, Farkas GL, Lovrekovich L (1964) Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54: 474–477

Kotzabasis K, Christakis-Hammas PD, Roubelakis-Angelakis KA (1993) A narrow-bore HPLC method for the identification and quantitation of free, conjugated, and bound polyamines. Anal Biochem 214: 484–489

Kroj T, Rudd JJ, Nurnberger T, Gabler Y, Lee J, Scheel D (2003) Mitogen-activated protein kinases play an essential role in oxidative burst-independent expression of pathogenesis-related genes in parsley. J Biol Chem 278: 2256–2264

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48: 251–275

Lazzarato L, Trebbi G, Pagnucco C, Franchin C, Torrigiani P, Bettì L (2009) Exogenous spermidine, arsenic and β-amaminobutyric acid modulate tobacco resistance to tobacco mosaic virus, and affect local and systemic glucosylsaccharic acid levels and arginine decarboxylase gene expression in tobacco leaves. J Plant Physiol 166: 90–100

Miller JH (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Mitsuya Y, Takahashi Y, Berberich T, Miyazaki A, Matsumura H, Takahashi H, Terauchi R, Tomonobu K (2009) Spermine signaling plays a significant role in the defense response of Arabidopsis thaliana to cucumber mosaic virus. J Plant Physiol 166: 626–643

Moschou PN, Delis ID, Paschalidis KA, Roubelakis-Angelakis KA (2008a) Transgenic tobacco plants overexpressing polyamine oxidase are not able to cope with oxidative burst generated by abiotic factors. Physiol Plant 133: 140–156

Moschou PN, Paschalidis KA, Delis ID, Andriopoulou AH, Lagiotti GD, Yakoumakis D1, Roubelakis-Angelakis KA (2008b) Spermidine exodus and oxidation in the apoplast induced by abiotic stress is responsible for H2O2 signatures that direct tolerance responses in tobacco. Plant Cell 20: 1708–1724

Moschou PN, Paschalidis KA, Roubelakis-Angelakis KA (2008c) Plant polyamine catabolism: the state of the art. Plant Signal Behav 3: 1061–1066

Moschou PN, Sammartin M, Andriopoulou AH, Rojo E, Sanchez-Serrano JJ, Roubelakis-Angelakis KA (2008d) Bridging the gap between plant and mammalian polyamine catabolism: a novel peroxosomal polyamine oxidase responsible for a full back-conversion pathway in Arabidopsis. Plant Physiol 147: 1845–1857

Negrel J, Vallee JN, Martin C (1984) Ornithine decarboxylase activity and the hypersensitive reaction of tobacco to tobacco mosaic virus in Nicotiana tabacum. Cucumber 23: 2747–2751

Palukaitis P, Roossinck MJ, Dietzgen RG, Franci R (1992) Cucumber mosaic virus. Adv Virus Res 41: 281–348

Paschalidis KA, Roubelakis-Angelakis KA (2005a) Spatial and temporal distribution of polyamine levels and polyamine anabolism in different organs/tissues of the tobacco plant: correlations with age, cell division/expansion, and differentiation. Plant Physiol 138: 142–152

Paschalidis KA, Roubelakis-Angelakis KA (2005b) Sites and regulation of polyamine catabolism in the tobacco plant: correlations with cell division/expansion, cell cycle progression, and vascular development. Plant Physiol 138: 2174–2184

Primikirios NI, Roubelakis-Angelakis KA (2001) Indications for post-translational regulation of Vitis vinifera L. arginine decarboxylase. Plant Mol Biol 45: 669–678

Rea G, de Pinto MC, Tavazza R, Biondi S, Gobbi V, Ferrante P, De Gara L, Federico R, Angelini R, Tavladoraki P (2004) Ectopic expression of Arabidopsis thaliana copper amine oxidase responsible for a full back-conversion pathway in Arabidopsis. Mol Biol 3350–3356

Ryals JA, Nevenschander UH, Willits MG, Molina A, Steiner HY, Hunt JM (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819

Samuel MA, Ellis BE (2002) Double jeopardy: both overexpression and suppression of a redox-activated plant mitogen-activated protein kinase render tobacco plants ozone sensitive. Plant Cell 14: 2059–2069

Sharma PC, Ito ZA, Shimizu ZT, Terauchi ZR, Kamoun S, Saitoh ZH (2003) Virus-induced silencing of WIPK and SIPK genes reduces resis-

1980 Plant Physiol. Vol. 149, 2009
tance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in Nicotiana benthamiana. Mol Genet Genomics 269: 583–591

Spurr AR (1969) A low viscosity embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43

Takahashi Y, Berberich T, Miyazaki A, Seo S, Ohashi Y, Kusano T (2003) Spermine signalling in tobacco: activation of mitogen-activated protein kinases by spermine is mediated through mitochondrial dysfunction. Plant J 36: 820–829

Tsiamis G, Mansfield JW, Hockenhull R, Jackson RW, Sesma A, Athanassopoulos E, Bennet MA, Stevens C, Vivian A, Taylor JD, et al (2000) Cultivar specific avirulence and virulence functions assigned to avrPphF in Pseudomonas syringae pv phaseolicola, the cause of bean halo-blight disease. EMBO J 19: 3204–3214

Vivian A, Arnold DL (2000) Bacterial effector genes and their role in host-pathogen interactions. J Plant Pathol 82: 163–178

Walters DR (2003) Polyamines and plant disease. Phytochemistry 64: 97–107

Yamakawa H, Kamada H, Satoh M, Ohashi Y (1998) Spermine is a salicylate-independent endogenous inducer for both tobacco acidic pathogenesis-related proteins and resistance against tobacco mosaic virus infection. Plant Physiol 118: 1213–1222

Yasuda M, Nakashita H, Hasegawa S, Nishioka M, Arai Y, Uramoto M, Yamaguchi I, Yoshiida S (2003) N-Cyanomethyl-2-chloroisonicotinamide induces systemic acquired resistance in Arabidopsis without salicylic acid accumulation. Biosci Biotechnol Biochem 67: 322–328

Yoda H, Hiroi Y, Sano H (2006) Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. Plant Physiol 142: 193–206

Yoda H, Yamaguchi Y, Sano H (2003) Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. Plant Physiol 132: 1973–1981

Zhang S, Klessig DF (1998) The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. Proc Natl Acad Sci USA 95: 7225–7230