Disruption of Poly(ADP-ribosyl)ation Mechanisms Alters Responses of Arabidopsis to Biotic Stress

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Poly(ADP-ribosyl)ation is a posttranslational protein modification in which ADP-ribose (ADP-Rib) units derived from NAD⁺ are attached to proteins by poly(ADP-Rib) polymerase (PARP) enzymes. ADP-Rib groups are removed from these polymer chains by the enzyme poly(ADP-Rib) glycohydrolase (PARG). In animals, poly(ADP-ribosyl)ation is associated with DNA damage responses and programmed cell death. Previously, we hypothesized a role for poly(ADP-ribosyl)ation in plant defense responses when we detected defense-associated expression of the poly(ADP-ribosyl)ation-related genes PARG2 and NUDT7 and observed altered callose deposition in the presence of a chemical PARP inhibitor. The role of poly(ADP-ribosyl)ation in plant defenses was more extensively investigated in this study, using Arabidopsis (Arabidopsis thaliana). Pharmacological inhibition of PARP using 3-aminobenzamide perturbs certain innate immune responses to microbe-associated molecular patterns (flg22 and elf18), including callose deposition, lignin deposition, pigment accumulation, and phenylalanine ammonia lyase activity, but does not disrupt other responses, such as the initial oxidative burst and expression of some early defense-associated genes. Mutant parg1 seedlings exhibit exaggerated seedling growth inhibition and pigment accumulation in response to elf18 and are hypersensitive to the DNA-damaging agent mitomycin C. Both parg1 and parg2 knockout plants show accelerated onset of disease symptoms when infected with Botrytis cinerea. Cellular levels of ADP-Rib polymer increase after infection with avirulent Pseudomonas syringae pv tomato DC3000 avrRpt2, and pathogen-dependent changes in the poly(ADP-ribosyl)ation of discrete proteins were also observed. We conclude that poly(ADP-ribosyl)ation is a functional component in plant responses to biotic stress.

Current models for the overall organization of plant immune systems include preformed defenses and infection-induced basal and R gene-mediated defenses (Jones and Dangl, 2006; Bent and Mackey, 2007; McDowell and Simon, 2008). Basal immune responses are mediated by receptors that recognize ubiquitously expressed, highly conserved microbe-associated molecular patterns (MAMPs) such as bacterial flagellin or EF-Tu proteins or fungal chitin. Many pathogens express effector proteins that suppress basal host immune responses, but R gene-mediated defenses can be activated when host R proteins recognize the presence or activity of specific pathogen effectors (also called avirulence [avr] proteins). R gene activation usually induces a rapid, multifactor defense, including a programmed cell death response known as the hypersensitive response. Both basal and R gene-mediated defenses can engage protein phosphorylation, ion fluxes, reactive oxygen species (ROS) production, and production of defense signaling compounds such as salicylic acid (SA), nitric oxide, ethylene, and jasmonic acid (Fey and Parker, 2000; Hammond-Kosack and Parker, 2003). These signals, among other things, induce the expression of defense-associated genes and microRNAs that promote antimicrobial functions and protect the cell from its own defense systems.

One prominent cellular response to pathogen infection is cell wall reinforcement, which can prevent further ingress of the pathogen and also restrict the passage of nutrients and water (Grant and Mansfield, 1999; Lee et al., 2001). Cell wall reinforcement in response to pathogens includes the formation of cell wall appositions, or papillae, containing Hyp-rich glycoproteins, phenylpropanoid compounds such as monolignols, and callose (Bestwick et al., 1995, 1997; Soylu et al., 2005; Underwood and Somerville, 2008). Hydrogen peroxide and other ROS, often derived from NADPH oxidase complexes and/or peroxidase activity at sites of papilla formation, contribute to cross-linking of proteins and phenolics at the cell wall, resulting in a structurally reinforced cell wall (Bestwick

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et al., 1997; Thordal-Christensen et al., 1997; Brown et al., 1998; Soylu et al., 2005). Some bacterial and oomycete effectors suppress callose deposition as a virulence mechanism (Hauck et al., 2003; De Roy et al., 2004; de Torres et al., 2006; Sohn et al., 2007).

Previously, we found a poly(ADP-Rib) glycohydrolase (PARG2) and a Nudix hydrolase active on ADP-Rib and NADH (NUDT7) among a small group of less than 40 genes significantly up-regulated in multiple Ravn interactions between Arabidopsis (Arabidopsis thaliana) and Pseudomonas syringae pv tomato DC3000 (Pst DC3000; Adams-Phillips et al., 2008). nudt7 plants were more resistant to virulent and avirulent Pst DC3000 (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008) and also displayed a greatly reduced hypersensitive response to avirulent Pst DC3000 (Adams-Phillips et al., 2008). We also found that pharmacological inhibition of poly(ADP-Rib) polymerase (PARP) blocked the formation of callose-containing cell wall depositions induced by the MAMPs flg22 and elf18 (Adams-Phillips et al., 2008). This suggested a role for poly(ADP-ribosylation) in the pathways that regulate pathogen-elicited callose deposition and plant innate immune responses.

Poly(ADP-ribosylation) is an important posttranslational modification in many eukaryotes (Otto et al., 2005; Hassa and Hottiger, 2008). It is biochemically and functionally distinct from mono-ADP-ribosylation. At the organismal level, poly(ADP-ribosylation) in animals contributes to the pathology of stroke, ischemia, heart attack, and chemotherapy (Jagtap and Szabo, 2005). Poly(ADP-ribosylation) is carried out by PARPs, which use NAD+ as a substrate to catalyze both the attachment and elongation of ADP-Rib polymers on acceptor proteins. Automodified PARP and other poly (ADP-ribosylated) nuclear proteins (Hulet sky et al., 1989) can affect chromatin structure, transcription, replication, and DNA repair processes through PARP-mediated recruitment of other proteins (Masson et al., 1998; Simbulan-Rosenthal et al., 1999; Ahel et al., 2009). Therefore, PARP can act as a DNA damage sensor (Petrucco, 2003; Schreiber et al., 2006; Roldan-Arjona and Ariza, 2009). In addition, PARP and poly (ADP-ribosylation) can regulate cellular processes by modulating cellular levels of NAD+. Strong PARP activation can cause massive consumption of NAD+, which can alter cellular reduction/oxidation states, impact nicotinamide levels, and induce ATP depletion (Hashida et al., 2009).

Eukaryotic organisms (excluding yeast) express multiple PARP proteins, all bearing a conserved C-terminal PARP catalytic domain. The Arabidopsis genome encodes at least three putative PARPs (Hunt et al., 2004; Otto et al., 2005). Use of pharmacological PARP inhibitors is a common way of overcoming such potential functional redundancy and also allows conditional inactivation of PARP activity. 3-Aminobenzamide (3AB) is a widely used PARP inhibitor in both animal (Bryant et al., 2005; Beauchamp et al., 2009; Ding et al., 2009; Hernandez et al., 2009) and plant (Phillips and Hawkins, 1985; Berglund et al., 1996; Amor et al., 1998; Tian et al., 2000; Adams-Phillips et al., 2008; Ishikawa et al., 2009) studies and has been shown to inhibit plant PARP enzymatic activity (Chen et al., 1994; Babychuk et al., 1998). 3AB has also been used to demonstrate the linkage between PARP and PARC activity in plants. For example, application of 3AB restored wild-type levels of ADP-Rib polymer in Arabidopsis parg1 (tej) mutant plants that otherwise accumulate 5-fold higher levels of poly(ADP-Rib) than wild-type seedlings (Panda et al., 2002).

PARC hydrolyzes the ADP-Rib polymers synthesized by PARP (Davidovic et al., 2001). As such, PARC is often thought to reverse, or counteract, PARP activity. PARC does not, however, restore the large amounts of NAD+ that can be consumed through PARP activity, and PARP’s activity can increase cellular pools of free ADP-Rib, a known cell death signal in mammalian cells (Andrabi et al., 2006). Hence, PARC can either counteract or further contribute to the impacts of PARP activation, depending on cellular context. Known animal genomes encode a single PARC gene (Ame et al., 1999), and mutation of PARC leads to the accumulation of toxic ADP-Rib polymers and is lethal in mice and Drosophila (Hanai et al., 2004; Koh et al., 2004). Arabidopsis is a rare example of a eukaryote with two PARC genes, which are present due to a gene duplication (At2g31865 and At2g31870). Much less is known about the functional role of PARC in plants, but it has been shown that PARC1 plays a role in regulating circadian rhythms in Arabidopsis (Panda et al., 2002).

Free ADP-Rib (which is generated by PARP) is rapidly degraded to AMP by certain nudix hydrolase (NUDT) enzymes, including Arabidopsis NUDT2 and NUDT7 (Ogawa et al., 2005). ADP-Rib-specific nudix hydrolases are thought to have multiple roles: they (1) reduce the high levels of toxic free ADP-Rib, (2) reestablish energy levels by supplying a source for ATP, and (3) contribute to NAD+ maintenance (Rossi et al., 2002; Ogawa et al., 2005, 2009; Ishikawa et al., 2009). As noted above, multiple groups have identified impacts of Arabidopsis nudt7 mutants on responses to pathogens (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008).

There is evidence that plant PARPs are structurally and functionally homologous to mammalian PARP proteins (Chen et al., 1994; O’Farrell, 1995; Babychuk et al., 1998; Doucet-Chabeaud et al., 2001). DNA damage induced by ionizing radiation activates Arabidopsis PARP1 and PARP2 gene expression (Doucet-Chabeaud et al., 2001). Application of 3-methoxybenzamide, a chemical PARP inhibitor, alters rates of homologous recombination in Arabidopsis and tobacco (Nicotiana tabacum) plants (Puchta et al., 1995), further suggesting a role for poly(ADP-ribosylation) in DNA repair in plants. Accumulating evidence suggests that poly(ADP-ribosylation) is an important part of the plant...
response to abiotic stress (De Block et al., 2005; Vanderauwera et al., 2007). For example, parp1/parp2 double knockout Arabidopsis plants display increased resistance to drought, high light, and oxidative stresses (De Block et al., 2005), and PARP inhibitors such as 3AB protect soybean (Glycine max) and tobacco cell suspensions from oxidative and heat shock-induced programmed cell death (Amor et al., 1998; Tian et al., 2000). 3AB was also shown to inhibit oxidative stress-induced Phe ammonia lyase (PAL) activity in Catharanthus roseus tissue culture (Berglund et al., 1996).

Given the demonstrated roles of PARP in plant abiotic stress responses (De Block et al., 2005; Vanderauwera et al., 2007) and in animal cell stress and cell death (Heeres and Hergenrother, 2007; David et al., 2009), poly(ADP-ribosyl)ation has received surprisingly little research attention regarding plant immunity and biotic stress responses. In this study, we used a combination of PARP inhibitors, genetic mutant analysis, and biochemical assays to further dissect the role of poly(ADP-ribosyl)ation in plant-pathogen interactions. We determined that although PARP inhibition and PARG gene disruption do not disrupt initial responses such as ROS production, they impact several MAMP-triggered responses downstream of ROS production, including callose and lignin deposition and phenylpropanoid pathway activation, and can accelerate the onset of the symptoms caused by the necrotrophic pathogen Botrytis cinerea. We also detected changes in the abundance of ADP-Rib polymers and poly(ADP-ribosyl)ated proteins during various Arabidopsis-pathogen interactions. These data provide further evidence that poly(ADP-ribosyl)ation plays significant, diverse roles in the coordination of plant responses to biotic stress.

RESULTS
PARP Inhibitor Impacts MAMP-Induced Plant Responses Downstream of ROS Production

Our previous study revealed that pharmacological inhibition of PARP blocks callose-containing cell wall depositions induced by either flg22 or elf18 in Arabidopsis (Adams-Phillips et al., 2008). As a follow-up to these experiments, we examined the effects of PARP inhibitor on other instances of induced callose production. We discovered that treatment with the PARP inhibitor 3AB does not block wound-induced callose deposition, nor does it reduce the constitutively active callose observed in mekk1 mutants (Suarez-Rodriguez et al., 2007; Fig. 1A), indicating that 3AB specifically blocks MAMP-induced callose deposition. It is also unlikely that PARP inhibitor directly impacts the PMR4 callose synthase enzyme that is responsible for most MAMP- and wound-induced callose synthesis (Jacobs et al., 2003; Nishimura et al., 2003; Kim et al., 2005; Soylu et al., 2005), as no alteration to wound-induced callose was observed when 3AB was added (Fig. 1A). In general, we found that a higher dose of 3AB was required to block elf18-induced callose deposition than for flg22-induced callose deposition (Supplemental Fig. S1).

To investigate where in the pathway of basal immune signaling 3AB might be acting to disrupt callose deposition, we examined the impact of PARP inhibition on MAMP-elicited production of ROS and induction of two MAMP-induced genes, WRKY29 and FRK1, that are normally expressed within 30 min of MAMP treatment (Asai et al., 2002). We found that flg22- or elf18-induced ROS production and WRKY29 and FRK1 gene expression were not significantly altered by 3AB treatment (Fig. 1, B and C). We also observed that 3AB can inhibit callose production when seedlings are treated with PARP inhibitor at 5 h, but not at 24 h, after elicitation with MAMPs (Fig. 1D), supporting the hypothesis that the blockage of callose deposition by PARP inhibitor is independent of early MAMP responses such as ROS production and induction of WRKY29 and FRK1 gene expression.

Callose Deposition Blocked by PARP Inhibitor Treatment Can Be Rescued by SA

We further investigated how 3AB may be acting to block elf18- and flg22-induced callose production, as relatively little is known about the pathways that lead to MAMP-induced callose deposition. Clay et al. (2009) recently demonstrated that flg22-induced callose requires induction of multiple pathways, including an ethylene/MYB51-dependent indole-3-glucosinolate biosynthesis pathway and a CYP81F2-dependent pathway. Therefore, we tested the impact of PARP inhibition on additional aspects of the MAMP-induced callose pathway. PARP inhibition by 3AB treatment did not alter flg22-induced MYB51 or CYP81F2 gene expression (Fig. 2A). These results suggest that 3AB blockage of MAMP-induced callose may be independent of and/or downstream of the MYB51/ethylene-dependent and CYP81F2/13G pathways. It has been reported that defects in defense-associated callose deposition in pen2, pcs1, and vtc1 mutants can be rescued by SA treatment (Clay et al., 2009). We found that, although treatment of seedlings with only SA or benzothiadiazole (BTH; a chemical analog of SA) does not induce callose deposition, addition of SA or BTH to flg22-treated seedlings can rescue 3AB blockage of flg22-induced callose deposition (Fig. 2B). Furthermore, use of an npr1 mutant revealed that this rescue by SA of 3AB blockage of callose is independent of NPR1 (Table I). These results could suggest that PARP inhibition interferes with a SA-dependent, NPR1-independent callose pathway upstream of SA biosynthesis or, alternatively, that in the presence of flg22, exogenous application of SA or BTH can activate an independent pathway and bypass the flg22-induced callose deposition pathway that is blocked by PARP inhibition. Callose deposition was still elicited by flg22 treatment in
nahG* (salicylate-degrading) and sid2* (salicylate biosynthesis-defective) plants that have greatly reduced SA production (Table I), as was also observed by Clay et al. (2009), which suggests that SA is not required for flg22-induced callose deposition. The above results further define the complex regulatory network that controls pathogen-responsive callose deposition (see “Discussion”).

PARP Inhibitor Disrupts Aspects of the Phenylpropanoid Pathway

In addition to our experiments with callose deposition, we examined the effects of PARP inhibitor on lignin deposition, a very different type of pathogen-induced cell wall reinforcement. Lignin is polymerized from soluble phenolics that, along with callose, can be found in pathogen-induced papillae (Lawton and Lamb, 1987; Nicholson and Hammerschmidt, 1992; Bhuiyan et al., 2009). We found that treatment with 3AB reduced elf18-induced guaiacyl lignin accumulation (Fig. 3A). Blockage of elf18-induced guaiacyl lignin is independent of the ability to induce callose deposition; pmr4 mutants, which are deficient in MAMP-induced callose deposition (Kim et al., 2005), still produce MAMP-elicited lignin (Fig. 3B).

Throughout the course of our experiments with elf18-treated seedlings, we observed that treatment with elf18 peptide elicits the accumulation of a dark brown pigment in the cotyledons of seedlings after several days of growth in liquid medium (Fig. 3C). Treatment of seedlings with L-α-aminooxy-β-phenylpropionic acid (AOPP), a chemical inhibitor of PAL activity (Kudakasseril and Minocha, 1986; Prats et al., 2007; Pan et al., 2008), reduces the accumulation of this pigment (Fig. 3D), suggesting that this pigment is likely a product of the phenylpropanoid pathway. In further support of this, seedlings with a mutation in

![Figure 1.](image-url)
the chalcone synthase gene, a key regulator in the production of pigments from the phenylpropanoid pathway, did not accumulate this pigment (data not shown). Interestingly, treatment with PARP inhibitor can block this pigment from accumulating in wild-type seedlings (Fig. 3D), indicating that 3AB may act to inhibit elf18-induced activation of the phenylpropanoid pathway, resulting in reduced pigment and lignin formation. Treatment of seedlings with 3AB significantly reduced PAL activity in elf18-elicited seedlings (Fig. 3E), supporting the Berglund et al. (1996) result that 3AB inhibits PAL activity in C. roseus tissue culture protein extracts in response to oxidative stress. Together with the blocked callose deposition, these results indicate that poly(ADP-ribosyl)ation processes engaged during plant defense may contribute to the regulation of multiple components deposited in pathogen-elicited plant cell wall modifications.

Disruption of PARG1 Gene Expression Exacerbates a Subset of MAMP-Triggered Plant Responses

Previously, we demonstrated that PARG2 (At2g31865) gene expression was up-regulated in response to flg22 treatment and during incompatible and compatible interactions with Pst DC3000 and its derivatives (Adams-Phillips et al., 2008). In this study, the regulation of PARG2 during plant defense responses was further investigated. A robust up-regulation of PARG2 gene expression was observed upon infection with the necrotrophic fungus B. cinerea and in the constitutive defense mutants nudt7 and cpr5-2 (Supplemental Fig. S2), supporting a role for PARG2 in general plant defense responses. On the other hand, PARG1 (At2g31870) gene expression was not significantly induced by B. cinerea (Supplemental Fig. S2) and was transiently induced 30 min after MAMP treatment (Supplemental Fig. S2).

T-DNA insertion lines disrupting the PARG1 and PARG2 genes were acquired, and reverse transcription (RT)-PCR was used to confirm reduction in expression of RNA for the appropriate loci (Supplemental Fig. S3). Similar to experiments with 3AB, we found that ROS production is not altered in parg mutants (Supplemental Fig. S3). MAMP-induced lignin and callose production also were not noticeably altered in parg mutants (data not shown). Seedling growth inhibition is used as a marker of innate immune responses in plants (Gomez-Gomez et al., 1999); growth inhibition is common in plants that have continuously activated defenses (Greenberg and Ausubel, 1993; Bowling et al., 1994; Yu et al., 1998). Notably, the response of parg1 mutant plants to elf18 peptide in seedling growth inhibition assays is stronger (Fig. 4A), analogous to the exaggerated response to flg22 seen in wild-type seedlings treated with 3AB (Adams-Phillips et al., 2008). This was observed for two independent T-DNA insertion lines representing two different parg1 mutant alleles (Supplemental Fig. S3). Coincident with the exaggerated seedling growth inhibition response, more elf18-induced pigment accumulates in parg1 mutants compared with wild-type seedlings (Fig. 4B). It is also

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**Table 1.** Callose response of Arabidopsis seedlings after flg22 treatment, with or without 3AB and SA treatment

<table>
<thead>
<tr>
<th>Genotype</th>
<th>flg22</th>
<th>flg22 + 3AB</th>
<th>flg22 + 3AB + SA</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>nphG*</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>sidi2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>apr1</td>
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+ Extensive callose deposition; –, little or no callose deposition. Callose deposition was monitored by microscopic analysis after aniline blue staining in seedlings collected and fixed 24 h after flg22 treatment.
notable that \textit{parg1} seedlings are hyperresponsive to the DNA-damaging agent mitomycin C (Fig. 4C). Even though \textit{PARG2} (and not \textit{PARG1}) expression is strongly induced by flg22 or elf18 treatment (Supplemental Figs. S2 and S3), it is the \textit{parg1} mutant that exhibited the above alterations in response to defense-eliciting MAMPs. \textit{parg2} mutants exhibited responses to elf18 and flg22 elicitation as well as to mitomycin C treatment that were not distinguishable from the response of wild-type plants (Fig. 4).

Disruption of \textit{PARG} Gene Expression Potentiates Arabidopsis Susceptibility to the Necrotrophic Pathogen \textit{B. cinerea}

In order to further characterize the role of \textit{PARG} genes in plant defense responses, \textit{parg} mutants were tested for altered susceptibility to pathogens. No significant differences between the wild type and \textit{parg} mutants were observed in limiting the growth of virulent and avirulent \textit{Pst} DC3000 (Supplemental Fig. S4). In an experiment with multiple replicates but that to date has been performed only once, we also did not observe any alteration in the macroscopic hypersensitive response in \textit{parg} mutants in response to avirulent \textit{Pst} DC3000 or in 3AB-treated leaves in response to dexamethasone-induced expression of \textit{avrRpt2} (Supplemental Table S1). However, in multiple experiments, both \textit{parg1} and \textit{parg2} knockdown plants displayed an accelerated onset of symptoms relative to wild-type plants after spray inoculation with \textit{B. cinerea} spores (Fig. 5). This increased susceptibility was statistically significant, although not as severe as the susceptibility of \textit{ein2-1} mutant plants (Fig. 5), which

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{PARP inhibitor also disrupts MAMP-induced lignification and other aspects of the phenylpropanoid pathway. A, Guaiacyl lignin formation in wild-type Col-0 Arabidopsis seedlings mock treated (0.6\% DMSO and distilled, deionized water) or treated with 2.5 \textit{mM} 3AB followed by either distilled, deionized water or 0.5 \textit{mM} elf18 for 48 h, then fixed and stained with phloroglucinol. B, Guaiacyl lignin in Arabidopsis wild-type (Col), \textit{efr} mutant (EF-Tu insensitive), and \textit{pmr4} mutant (callose synthase) seedlings exposed to elf18 elicitor 48 h prior to staining as in A. C, Dark pigments in wild-type seedlings treated with distilled, deionized water (ddH2O) or 2.5 \textit{mM} elf18 for 5 d. Leaves in the top panels were photographed in natural light (no fixing or staining), and leaves in the bottom panels were cleared in ethanol and then the area of leaf blade near the petiole was photographed (view is of approximately 100 leaf cells). D, Dark pigments in seedlings mock treated with 0.6\% DMSO, 0.1 \textit{mM} AOPP (PAL inhibitor) and 0.6\% DMSO, or 2.5 \textit{mM} 3AB prior to treatment with 2.5 \textit{mM} elf18 for 5 d, at which time leaves were cleared in ethanol and photographed. In A to D, photographs are representative of multiple replicate samples, and the experiments shown were repeated at least twice with similar results. E, PAL activity in 10-d-old Arabidopsis seedlings treated with 2.5 \textit{mM} elf18 and/or 5 \textit{mM} 3AB. PAL activity was measured after 24 h. The graph presents results from three independent biological replicates (means \pm se). Bars with the same letter are not significantly different (one-way ANOVA; Tukey’s simultaneous test; \( P < 0.001 \)).}
\end{figure}
are known to be hypersusceptible to this fungus (Thomma et al., 1999).

Interaction of Adult Arabidopsis with Pst DC3000 Leads to Activation of Cellular Poly(ADP-ribosyl)ation Reactions

In addition to inhibitor and mutant studies, direct biochemical assays were carried out to test for changes in poly(ADP-ribosyl)ation during plant responses to pathogens. Since PARP consumes NAD\(^+\) to synthesize ADP-Rib units, an examination of cellular NAD\(^+\) levels can be used as an indirect measure of poly(ADP-Rib) synthesis activities (Chen et al., 1994; Du et al., 2003; De Block et al., 2005; Ishikawa et al., 2009). Whereas no significant change in NAD\(^+\) levels was seen for seedlings treated with avirulent pathogen, flg22 and/or 3AB (data not shown), a statistically significant 40% to 50% decrease in NAD\(^+\) compared with mock-infiltrated samples was observed in leaves 12 h after infiltration with virulent Pst DC3000 (Supplemental Fig. S5). The decrease in NAD\(^+\) concentrations observed by DeBlock et al. (2005) in Arabidopsis exposed to abiotic stresses such as high light was of a similar 50% magnitude. However, it is possible that the decrease in NAD\(^+\) that we observed reflects perturbations in basic cellular mechanisms due to the progression of successful infection by a virulent pathogen, rather than reflecting the activation of an NAD\(^+\)-consuming PARP enzymatic reaction. Therefore, we turned to immunodetection of poly(ADP-Rib) polymer levels as a more direct measure of PARP activation.

Poly(ADP-Rib) polymers and poly(ADP-ribosyl)ated protein species were monitored in seedlings treated with flg22 peptide as well as in adult Arabidopsis leaf tissue during interactions with virulent and avirulent Pst DC3000 strains and B. cinerea (Fig. 6; Supplemental Fig. S6). No significant changes in poly(ADP-Rib) levels were detected in flg22- or 3AB-treated seedlings (Supplemental Fig. S6), but both total cellular and nuclear poly(ADP-Rib) polymers increased somewhat in response to virulent Pst DC3000 and increased significantly (by 50%) in adult leaves treated with avirulent Pst DC3000 at 12 h post infection (hpi) relative to mock-treated leaves (Fig. 6A). DeBlock et al. (2005) observed a quantitatively similar increase in total polymer levels during abiotic (high-light) stress responses in Arabidopsis. We also observed a presence of elf18 or solvent control, then cleared in ethanol and photographed. C, Sensitivity to the DNA-damaging agent mitomycin C (MMC). Wild-type (Col-0), parg1-1, parg1-2, and parg2 seedlings were grown in the presence or absence of 40 μM mitomycin C for 10 d, and seedling weights were recorded. Asterisks summarize results across three experiments for ANOVA tests of similarity of means between wild-type and mutant plants for the same treatment (Tukey’s simultaneous test: \(P < 0.01\); ** \(P < 0.0001\). [See online article for color version of this figure.]
2-fold increase in poly(ADP-Rib) polymer levels in positive control experiments that used high-light stress (data not shown).

When Arabidopsis leaf extracts were separated by SDS-PAGE, a poly(ADP-ribosyl)ated species migrating at an apparent mass of 43 kD was reproducibly 2- to 5-fold more abundant in both compatible and incompatible interactions with Pst DC3000 at 4 hpi than in mock-treated tissue (Fig. 6, B and C). The presence of low levels of this modified protein in mock-inoculated samples (Fig. 6C) supports the notion that it is an endogenous plant protein and not a bacterial protein present in both compatible and incompatible interactions. The 43-kD band detected by immunodetection methods (Fig. 6C) was not abundant enough to yield sufficient protein for mass spectrometry characterization in scaled-up experiments. Conversely, the abundance of a poly(ADP-ribosyl)ated doublet (approximately 50 kD) dramatically decreased over the first 2 d of infection with B. cinerea (Fig. 6D).

Individually, poly(ADP-ribosyl)ated proteins were also monitored by SDS-PAGE in 14-d-old seedlings treated with flg22 or 3AB. No reproducibly detectable changes to individual modified protein species were observed, although the number of apparent poly(ADP-ribosyl)ated proteins was consistently much greater in seedlings than in adult leaves (Supplemental Fig. S6).

**DISCUSSION**

In this study, we used a combination of pharmacological inhibitors, genetic mutants, and biochemical assays to examine the role of poly(ADP-ribosylation) during plant innate immune responses to MAMPs, biotrophic bacteria, and a necrotrophic fungus.

**Poly(ADP-ribosylation) Regulates a Subset of Basal Immune Responses**

While callose and lignin deposition responses are reduced after 3AB treatment, other MAMP-induced responses, such as ROS production and WRKY29 and FRK1 gene expression, remained unchanged (Fig. 1, B and C). Likewise, our analysis of parg mutants indicated that while knockout of parg1 leads to hypersensitivity to elf18 treatment (exacerbated seedling growth inhibition and increased pigment production; Fig. 4, A and B), other MAMP-triggered responses such as the early ROS burst were not affected (Supplemental Fig. 3C). These and other findings demonstrate that poly(ADP-ribosylation) regulates a subset of plant basal immune responses.

As is also found in the animal literature (see introduction), during plant defense responses PARP seemingly acted to enhance the impacts of PARP activity or to counteract the impacts of PARP activity, depending on cellular context. 3AB treatment and parg1 mutation both caused exaggerated seedling growth inhibition upon MAMP treatment, yet 3AB (and not parg1 mutation) disrupted callose and lignin deposition, and parg1 mutation (but not 3AB) caused elevated pigment production in response to elf18.

**Poly(ADP-ribosylation) and MAMP-Elicited Plant Cell Wall Modifications**

We previously reported that PARP inhibition blocks MAMP-induced callose deposition (Adams-Phillips et al., 2008). In this study, we found that PARP inhibition by 3AB specifically inhibits MAMP-elicited callose and not wound-induced callose or callose produced in mekk1 mutants (Fig. 1). 3AB still blocked callose deposition if applied 5 h after initiation of innate immune signaling events. 3AB also blocked the production of lignin (Fig. 3A), a product of the phenylpropanoid pathway and another key component of pathogen-induced papillae. PARP inhibition also blocked production of an elf18-induced pigment that is presumed to derive in part from the phenylpropanoid pathway (since AOPP, a PAL inhibitor, also blocks this pigment production; Fig. 3D). We further determined that 3AB treatment can block activation of PAL in intact MAMP-elicited seedlings (Fig. 3E). PAL controls one of the first committed steps in the phenylpropanoid pathway, indicating that PARP likely has a global impact on numerous pathogenesis-induced products from the phenylpropanoid pathway.

This study contributes additional insight into the signaling networks that regulate MAMP-induced callose deposition (Kim et al., 2005; Clay et al., 2009). We found that the predominant signaling network that mediates flg22-elicited callose deposition is blocked by...
PARP inhibitor. That network apparently can function independent of SA because flg22-induced callose deposition still occurs in sid2 mutants or in plants expressing nahG as well as in npr1 mutants (Table I). However, there appears to be a flg22-responsive branch in the callose signaling pathway that potentiates SA-responsive callose deposition. SA alone does not induce callose deposition, but flg22 + SA does, indicating that flg22 potentiates SA-responsive callose deposition. SA or BTH feeding can bypass 3AB inhibition of flg22-induced callose deposition, suggesting that 3AB does not block the flg22-potentiated SA-responsive branch of the network. Hence, it seems likely that there is a separate portion of the signaling network, leading from flg22 perception to potentiation of SA-responsive/NPR1-independent callose deposition, that PARP inhibitor does not block. PARP inhibition by 3AB also did not alter flg22-induced MYB51 or CYP81F2 gene expression (Fig. 2A), which Clay et al. (2009) had previously shown are induced as part of the distinct ethylene/MYB51-dependent and CYP81F2-dependent pathways that are required for flg22-induced callose deposition. Our data, therefore, additionally suggest that 3AB blockage of MAMP-induced callose is independent of and/or downstream of the MYB51/ethylene-dependent and CYP81F2/I3G-dependent portions of these networks.

We found that chemical PARP inhibition blocks components of the phenylpropanoid pathway, which raises experimentally challenging questions as to how this impacts responses to plant pathogens. There are two proposed pathways for SA biosynthesis in plants: through isochorismate synthase and through the phenylpropanoid pathway (Mauch-Mani and Slusarenko, 1996; Wildermuth et al., 2001). In future work, it may be of interest to test if PARP is impacting one or the other source of SA. Besides SA, other products of the phenylpropanoid pathway include ROS scavengers. Vitamin C-deficient mutants (vtc1) impaired in ROS scavenging activities exhibit reduced MAMP-induced callose production that can be rescued by SA treatment (Clay et al., 2009), similar to our experiments with 3AB (Fig. 2B). We observed no alteration in MAMP-elicited ROS production in the first half hour after treatment with PARP inhibitor (Fig. 1B), indicating that poly(ADP-ribosyl)ation does not regulate the deposition of cell wall reinforcement compounds such as callose and lignin by regulating early ROS burst events after defense elicitation. However, it remains possible that poly(ADP-ribosyl)ation can at some secondary or tertiary level alter defense-associated ROS levels and/or the response to those ROS, such as by causing shifts in phenylpropanoid metabolites that alter ROS scavenging.

Figure 6. Poly(ADP-ribosyl)ation is activated by pathogen attack. A, Total cellular ADP-Rib polymer abundance in Col-0 plants 12 h after infiltration with virulent (DC3000) or avirulent (DC3000 avrRpt2) Pst DC3000 relative to mock-treated (buffer) plants. ADP-Rib polymer levels were quantified by immuno-dot blot and image analysis software. Means ± se are shown for intensity levels normalized to intensity for mock-treated material within same experiment. * P < 0.001 for ANOVA (Tukey’s simultaneous test for similarity of means between pathogen-treated and mock-inoculated plants) for five separate experiments. B, Original image of a representative experiment, monitoring poly(ADP-ribosyl)ation of a 43-kD protein species detected by immunoblot (IB) analysis using anti-poly(ADP-Rib) antibody after Ponceau S (P) staining. C, Quantification for three immunoblot experiments (Tukey’s simultaneous test; * P < 0.001). D, SDS-PAGE and immunoblot (IB) analysis of poly(ADP-ribosyl)ated proteins in Col-0 plants sampled at 1 (lane 1), 2 (lane 2), and 3 (lane 3) d post inoculation (DPI) after spraying with 1 × 10⁶ spores ml⁻¹ B. cinerea. Blots were stained with Ponceau S before immunoblotting.

Activation of Cellular Poly(ADP-ribosyl)ation Reactions in Response to Pathogen Infection

When we examined total ADP-Rib polymer levels in plants inoculated with different strains of \textit{Pst DC3000}, we found a significant increase in total ADP-Rib polymer 12 h after infection in plants inoculated with avirulent but not virulent pathogen (Fig. 6A). This observation provides further evidence of the previously unknown association between poly(ADP-ribosyl)ation and plant responses to pathogens. Free ADP-Rib polymer is a known cell death signal in animal cells, acting at the mitochondria to stimulate release of apoptosis-inducing factor (Heeres and Hergenrother, 2007; David et al., 2009), but in initial experiments, we have not observed an overt change in the severity or rate of development of macroscopic \textit{avrRpt2}-elicited hypersensitive response symptoms in \textit{par1} and \textit{par2} mutants or with 3AB treatment (Supplemental Table S1). However, given the findings regarding apoptosis and poly(ADP-Rib) in animal systems, our detection of elevated ADP-Rib polymer during an incompatible interaction suggests that, in the future, a study of the possible role of ADP-Rib polymer in plants responding to avirulent pathogen may be warranted. In addition, although \textit{par} mutants showed no macroscopic changes in their interaction with biotrophic \textit{Pst DC3000} (Supplemental Fig. S4), these same mutants were more susceptible to the necrotrophic pathogen \textit{B. cinerea} (Fig. 5). Arabidopsis \textit{par} mutants are known to accumulate ADP-Rib polymers (Panda et al., 2002), but future studies will be required to investigate causal relationships of poly(ADP-Rib) polymer accumulation and the observed increase in susceptibility to necrotrophic pathogens.

We also observed significant accumulation of a discrete poly(ADP-ribosyl)ated protein species in response to virulent and avirulent \textit{Pst DC3000} at 4 hpi (Fig. 6, B and C). From these experiments, we conclude that poly(ADP-ribosyl)ation of at least one target protein occurs as an initial response to contact with \textit{Pst DC3000}. We also detected significantly decreased abundance of a poly(ADP-ribosyl)ated protein over the first 2 d of infection with \textit{B. cinerea} (Fig. 6D), indicating the dynamic nature of PARP activity during two very different types of plant-pathogen interactions. Despite the observed increase in ADP-Rib polymer and protein poly(ADP-ribosyl)ation in response to avirulent \textit{Pst DC3000}, NAD$^+$ levels at 4, 8, and 12 h did not detectably change in adult plants inoculated with avirulent pathogen (Supplemental Fig. S5). Depletion of NAD$^+$ pools is suggested to be significant in plant abiotic stress and for some animal systems (Du et al., 2003; De Block et al., 2005). We cannot exclude the possibility that the methods we used to detect NAD$^+$ and ADP-Rib polymer were not sensitive enough to detect more subtle or transient yet significant changes. However, the results of this study suggest that PARP activation does not affect plant defense through significant depletion of NAD$^+$ pools after activation of PARP enzyme.

NUDT7 Biotic Stress Findings Also Implicate Poly(ADP-ribosyl)ation

Recent findings from other studies have shown impacts of Arabidopsis NUDT7 on biotic stress responses (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008). Although not demonstrably tied to poly(ADP-ribosyl)ation at the time, those findings further suggest possible impacts of poly(ADP-ribosyl)ation on plant responses to pathogens, especially in light of this report and recent results showing direct impacts of NUDT7 on plant poly(ADP-ribosyl)ation (Ishikawa et al., 2009). However, because NUDT7 action has multiple physiological impacts (see introduction), there are varied mechanisms through which NUDT7 may be impacting plant responses to biotic stress, and only some of them directly involve poly(ADP-ribosyl)ation. For example, the defense phenotypes of \textit{nudt7} mutants may be due to accumulation of free ADP-Rib, which may induce stress responses, or may be due to alterations in NADH hydrolysis rather than ADP-Rib hydrolysis (Ge et al. 2007).

Poly(ADP-ribosyl)ation at the Intersection between Plant Defense and DNA Repair

In animal systems, PARP is most prominent as a DNA break sensor and DNA repair pathway signaling molecule. DNA strand breaks are known to activate the expression and activity of PARP enzymes in plants (Babiychuk et al., 1998; Doucet-Chabeaud et al., 2001; Chen et al., 2003). While pathogen-induced ROS production in plants contributes positively to disease resistance in a number of ways (Levine et al., 1994; Wojtaszek, 1997; Neill et al., 2002; Apel and Hirt, 2004), these same ROS can also oxidize DNA, creating a genotoxic challenge that the host must respond to. Therefore, by activating appropriate DNA repair pathways (Ishikawa et al., 2009) and protective mechanisms, poly(ADP-ribosyl)ation may be an important response to ROS production during defense. Phenylpropanoid pathway products can function in plant defense as ROS scavengers and as protective UV light-absorbing pigments or “sunscreen” that protect DNA from UV light-induced free radicals (superoxide, singlet oxygen, and hydroxyl radicals; Biezas and Lois, 2001; Filkowski et al., 2004; Ferrer et al., 2008). We observed that knockout of \textit{par1} leads to hyperaccumulation of a phenylpropanoid-derived pigment in response to elf18 treatment (Fig. 4B) and that these same mutants are also more sensitive to the DNA-damaging agent mitomycin C (Fig. 4C). It is possible, therefore, that there are interactions between poly(ADP-ribosyl)ation, regulation of phenylpropanoid pathway activity, and protection of the genome from genotoxic stress.

DNA repair pathways may also be engaged during plant defense responses for reasons other than as a response to genotoxic stress. Pathogen stresses, such as flg22 peptide and viral pathogen, increase somatic...
homologous recombination frequency and cause DNA breaks that require subsequent repair (Lucht et al., 2002; Kovalchuk et al., 2003; Molinier et al., 2006). These and other observations suggest a link between homologous recombination and effective plant defense (Durrant et al., 2007; Friedman and Baker, 2007). Since PARP is activated by DNA breaks, and because PARP inhibitor disrupts innate immune responses (as described here) and elicits somatic homologous recombination (Puchta et al., 1995; Lucht et al., 2002; Filkowski et al., 2004; A.G. Briggs and A.F. Bent, unpublished data), poly(ADP-ribosylation) may be involved in such recombination mechanisms.

In summary, this study shows that PARP inhibitors and parp mutants alter specific plant responses to elicitation by pathogens and that ADP-Rib polymer levels change during infection. Our results suggest that poly(ADP-ribosylation) is a component of the response to multiple different biotic stresses in plants. Poly(ADP-ribosylation) may contribute to protection against genotoxic stress, to genome recombination, or to pathogen-induced host cell death; these and other observations suggest a link between PARP responses (as described here) and biotic stress responses in plants, which may provide protective activities require further investigation. However, it is clear from the data presented that poly (ADP-ribosylation) is involved in defense-associated cell wall reinforcement and in the response to infection by B. cinerea.

**MATERIALS AND METHODS**

### Plant Lines and Growing Conditions

Arabidopsis (Arabidopsis thaliana accession Columbia [Col-0]) plants were grown at 22°C under short-day conditions (9 h of light/15 h of dark, 100–150 μmol m⁻² s⁻¹) at a density of 16 seeds per 81 cm². Aseptically grown Arabidopsis seedlings were obtained from surface-sterilized seeds germinated on 0.5× Murashige and Skoog agar medium with 2% (w/v) Suc and 1× Gamborg’s vitamins for 5 d. Seedlings were then transferred to liquid 0.5× Murashige and Skoog salts, 1.5% (w/v) Suc, and 1× Gamborg’s vitamins in 24-well plates for further analysis. The homozgyous T-DNA knockout lines par1-1 (SALK_147805), par2-1 (SALK_116088), parp1 (GABI_027280), and nudT7 (SALK_046410), all in the Col-0 background, were identified as described (Alonso et al., 2003; Rossano et al., 2003). *pmr4-1* (CS3858), *ciz2-1* (CS3071), *spr-2* (CS3770), and chalcone synthase tt-4 (CS85) mutant seeds were obtained from the Arabidopsis Biological Resource Center stock center; transgenic dexarvRpt2 plants (McNelis et al., 1998) were courtesy of B. Staskawicz (University of California-Berkeley), and *mekk1* seeds were kindly provided by P. Krysan (University of Wisconsin-Madison).

**Pst DC3000 Culture and Plant Inoculations**

*Pseudomonas syringae pv tomato* strain DC3000 carrying the plasmid pVSPl1 with no insert or with arvRpt2 under the control of its native promoter (Kunkel et al., 1993) was grown for 2 d on NYGA solid medium (5 g/L bactopeptone, 3 g/L yeast extract, 20 mL/L glycerol, and 15 g/L agar) at 28°C. Arabidopsis plants (4–6 weeks old) were vacuum infiltrated with bacteria resuspended in 10 mM MgCl₂ at 1 × 10⁷ colony-forming units mL⁻¹. Rosette leaves were collected by cutting with a razor at the basal stem at 4 hpi. Bacterial growth in leaves was quantified at 3 d post inoculation with 1 × 10⁷ colony-forming units mL⁻¹ using standard procedures (Suarez-Rodriguez et al., 2007). In each experiment, leaf punches from four leaves were pooled and tested by dilution plating for each data point, with four data points per treatment.

**RNA Extraction and Gene Expression Analysis**

Total RNA was extracted from leaf or seedling tissue (RNAeasy Plant Mini Kit; Qiagen). Contaminating DNA was removed with an RNase-free DNase set (Qiagen), and RNA concentrations were quantified by Nanodrop Spectrophotometer (Thermo Scientific). Semiquantitative RT-PCR was performed to be using a nonsaturating number of PCR cycles; reactions contained cDNA (synthesized with SuperScript III reverse transcriptase; Invitrogen), template, and 2× Taq polymerase (Fermentas). Primers were designed to correspond to homologous recombination frequency and cause DNA breaks that require subsequent repair (Lucht et al., 1995; Lucht et al., 2002). Since PARP is activated by DNA breaks, and because PARP inhibitor disrupts innate immune responses (as described here) and elicits somatic homologous recombination (Puchta et al., 1995; Lucht et al., 2002) and induce a localized wound response, cotyledons were punctured with a sharp needle. For callus analysis, seedlings were fixed in formaldehyde-acetic acid/ethanol (FAA) for 24 h, cleared in ethanol, and stained with 0.01% aniline blue as described (Gomez-Gomez et al., 1999). A minimum of 12 cotyledons per condition per experiment were visualized under UV light with an epifluorescence microscope. For guaiacyl lignin analysis, 3-d-old seedlings were fixed in FAA at 48 hpi, cleared in ethanol, and stained with 1:1 solution of 2% phloroglucinol and concentrated HCl, and photographed within 15 min of staining. For cell wall analysis, 6-d-old seedlings were fixed in FAA at 48 hpi, cleared in ethanol, stained with 1:1 solution of 2% phloroglucinol and concentrated HCl, and photographed within 15 min of phloroglucinol staining (Newman et al., 2004).

**Seeding Growth Inhibition Assays**

Seeds were treated with varying concentrations of elicitor, as described above, and fresh weight was recorded 10 to 14 d later for eight to 12 seedlings per treatment.

**Cell Wall Component Analysis**

One day after transfer to liquid medium, seedlings were treated with varying concentrations of different chemicals and MAMP elicitors as noted. To induce a broad wound response, cotyledons were squeezed with a pair of forceps. To induce a localized wound response, cotyledons were punctured with a sharp needle. For callose analysis, seedlings were fixed in formaldehyde-acetic acid/ethanol (FAA) for 24 h, cleared in ethanol, and stained with 0.01% aniline blue as described (Gomez-Gomez et al., 1999). A minimum of 12 cotyledons per condition per experiment were visualized under UV light with an epifluorescence microscope. For guaiacyl lignin analysis, 3-d-old seedlings were fixed in FAA at 48 hpi, cleared in ethanol, stained with 1:1 solution of 2% phloroglucinol and concentrated HCl, and photographed within 15 min of phloroglucinol staining (Newman et al., 2004).

**ROS Assay**

ROS were quantified using a luminol-based assay (Gomez-Gomez et al., 1999). Briefly, eight to 12 leaf discs per treatment were floated in 0.5% dimethyl sulfoxide (DMSO) overnight on a 96-well plate. Discs were treated with 0.6% DMSO or 2.5× 3AB for 30 min before addition of 0.1 mg mL⁻¹ luminol (Fluka) and 0.1 mg mL⁻¹ horseradish peroxidase (Sigma). Distilled, deionized water or 1.0 μM elf18 or flg22 was then added, and luminescence was measured approximately once per minute with a Synergy HT Multidetection Microplate Reader (BioTek). ROS data are presented as area under the curve for each disc normalized to the mean area for the control samples tested within the same experiment.

**Immunological Detection and Quantification of Poly(ADP-Rib)**

Concentrations of total protein (CellLytic P extraction buffer; Sigma) and nuclear protein (CellLytic PN kit; Sigma) extracts treated with 1:100 plant...
tissue culture protase inhibitor cocktail (Sigma) were quantified using bicinchonic acid protein assay reagents (Bio-Rad). Total poly(ADP-Rib) polymer was measured by dot blot as described (De Block et al., 2005; Hunt et al., 2007) using rabbit polyclonal anti-poly(ADP-Rib) primary antibody (Trevigen). Poly (ADP-ribosyl)ated proteins were analyzed by SDS-PAGE and immunoblot using the same rabbit polyclonal primary antibody, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad), and detected using enhanced chemiluminescence reagents (GE Healthcare). Equal gel loading was confirmed by Ponceau S (Sigma) staining prior to immunoblotting.

**NAD⁺ Quantification**

Total cellular NAD⁺ concentrations were quantified from adult leaf or seedling tissue using an alcohol dehydrogenase-based colorimetric enzyme cycling assay, as described (Jacobson and Jacobson, 1976). A purified NAD⁺ standard curve was used, and all data points were adjusted to total cellular protein concentrations, as determined by bicinchonic acid protein assay (Bio-Rad).

**PAL Activity Assays**

PAL activity was assayed as described (Olsen et al., 2008). Briefly, 30 10-d-old Arabidopsis seedlings were treated with inhibitor and/or elicitor as described and harvested at 24 hpi. Treated tissue was ground and passed through a Sephadex G-25 column (GE Healthcare Life Sciences). PAL activity was measured from eluate as L-Phe converted to trans-cinnamic acid per hour using Bradford assays (Sigma) were performed on extracts to quantify protein concentrations.

**Supplemental Data**

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

**Supplemental Figure S1.** Dose response of callose deposition in presence of PARG inhibitor.

**Supplemental Figure S2.** RT-PCR to monitor PARG gene expression.

**Supplemental Figure S3.** parg mutant characterization.

**Supplemental Figure S4.** Bacterial growth in parg mutants.

**Supplemental Figure S5.** NAD⁺ concentrations after infections by P. syringae pv tomato.

**Supplemental Figure S6.** Polymer levels and poly(ADP-ribose)lated proteins following biotic stress.

**Supplemental Table S1.** Hypersensitive response of parg mutants and in presence of PARP inhibitor.

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**LITERATURE CITED**


