Protein Poly(ADP-ribosyl)ation Regulates Arabidopsis Immune Gene Expression and Defense Responses

Baomin Feng1, Chenglong Liu2, Marcos V. V. de Oliveira1,2,3, Aline C. Intorne2,3, Bo Li2, Kevin Babilonia1,4, Gonçalo A. de Souza Filho4, Libo Shan2, Ping He1*

1 Department of Biochemistry and Biophysics, and Institute for Plant Genomics & Biotechnology, Texas A&M University, College Station, Texas, United States of America, 2 Department of Plant Pathology and Microbiology, and Institute for Plant Genomics & Biotechnology, Texas A&M University, College Station, Texas, United States of America, 3 Center of Biosciences and Biotechnology, Darcy Ribeiro State University of Northern of Rio de Janeiro, Brazil, 4 Department of Biology, University of Puerto Rico, Mayaguez Campus, Mayaguez, Puerto Rico

Abstract

Perception of microbe-associated molecular patterns (MAMPs) elicits transcriptional reprogramming in hosts and activates defense to pathogen attacks. The molecular mechanisms underlying plant pattern-triggered immunity remain elusive. A genetic screen identified Arabidopsis poly(ADP-ribose) glycohydrolase 1 (atparg1) mutant with elevated immune gene expression upon multiple MAMP and pathogen treatments. Poly(ADP-ribose) glycohydrolase (PARG) is predicted to remove poly(ADP-ribose) polymers on acceptor proteins modified by poly(ADP-ribose) polymerases (PARPs) with three PARPs and two PARGs in Arabidopsis genome. AtPARG1 and AtPARG2 possess poly(ADP-ribose) polymerase activity, and the activity of AtPARG2 was enhanced by MAMP treatment. AtPARG1, but not AtPARG2, carries glycohydrolase activity in vivo and in vitro. Importantly, mutation (G450R) in atparg1 blocks its activity and the corresponding residue is highly conserved and essential for human HsPARG activity. Consistently, mutant atparg1 atparg2 plants exhibited compromised immune gene activation and enhanced susceptibility to pathogen infections. Our study indicates that protein poly(ADP-ribose)lation plays critical roles in plant immune gene expression and defense to pathogen attacks.

Introduction

Plants sense the presence of pathogens by the cell-surface-localized pattern recognition receptors (PRRs), which perceive evolutionarily conserved pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), including bacterial flagellin, lipopolysaccharide (LPS), peptidoglycan (PGN), elongation factor Tu (EF-Tu), and fungal chitin [1–3]. A 22-amino-acid peptide corresponding to a region near the amino-terminus of flagellin (flg22) is recognized by the Arabidopsis PRR Flagellin-Sensing 2 (FLS2), a leucine-rich repeat receptor-like kinase (LRR-RLK) [4,5]. Perception of flg22 by FLS2 induces instantaneous massive transcriptional reprogramming [1–3]. It has been shown recently that BIK1 is able to phosphorylate plasma membrane-resident NADPH oxidase family member respiratory burst oxidase homolog D (RBOHD), thereby contributing to ROS production [20,21]. However, it remains largely unknown how PRR complex activation leads to profound immune gene transcriptional reprogramming.

Protein poly(ADP-ribose)ylation (PARylation), an important post-translational modification process, plays a crucial role in a broad array of cellular responses including DNA damage detection and repair, cell division and death, chromatin modification and gene transcriptional regulation [22–24] (S1 Fig.). PARylation is primarily mediated by members of poly(ADP-ribose) polymerases (PARPs), which transfer ADP-ribosyl moieties from nicotinamide adenine dinucleotide (NAD+) to different acceptor proteins at glutamate (Glu), aspartate (Asp) or lysine (Lys) residues resulting in
the formation of linear or branched poly(ADP-ribose) (PAR) polymers on acceptor proteins (S1 Fig.). PAR activities and PARPs have been found in a wide variety of organisms from archaea to mammals and plants, but they are apparently absent in yeast [25]. Human PARP-1 (HsPARP-1) is the most abundant and ubiquitous PARP among a family of 17 members, and it catalyzes the covalent attachment of PAR polymers on acceptor proteins (S2A Fig.) to PARylation and other target proteins, including histones, DNA repair proteins, transcription factors, and chromatin modulators [22]. HsPARP-1 possesses three functional domains with a DNA binding domain at N-terminus, auto-modification domain in the middle and a catalytic domain at C-terminus (S2A Fig.). PARylation is a reversible reaction and the covalently attached PAR on the target proteins can be hydrolyzed to free PAR or mono-(ADP-ribose) by poly-(ADP-ribose) glycohydrolase (PARG) [22,23] (S1 Fig.). PARG contains both endo- and exo-glycohydrolase activities that promote rapid catalytic destruction of PAR of target proteins [26]. There is only one PARG gene in humans with three different isoforms: PARGα and PARGβ in the cytoplasm and PARGγ in the nucleus [26]. Mammalian PARG possesses a regulatory and targeting domain (A-domain) at the N-terminus, a mitochondrial targeting sequence (MTS) in the middle and a conserved catalytic domain at the C-terminus [27] (S2B Fig.). The catalytic core containing “GGG-X6-8-QEE” PARG signature motif interacts with PAR and executes hydrolysis activity [28]. Despite of their apparently opposing activities, members of PARPs and PARGs coordinate regulate protein PARylation and play essential roles in a wide range of cellular processes and contribute to the pathogenicity of various diseases, including cancer, cardiovascular diseases, stroke, metabolic disorders, diabetes and autoimmunity [25].

The *Arabidopsis* genome encodes three members of PARPs, AtPARP1 (At2g31320), AtPARP2 (At4g02390) and AtPARP3 (At5g22470) and two members of PARGs, AtPARG1 (At2g31870) and AtPARG2 (At2g31865) [23,29] (S2A Fig.). AtPARP1 (it was originally named as AtpARP2) shares the conserved domain structure with HsPARP-1, whereas AtPARP2 (it was originally named as AtpARP1) and AtPARP3 more closely resemble HsPARP-2 and HsPARP-3 [29] (S2A Fig.). As their mammalian counterparts, plant PARPs are implicated in DNA repair, cell cycle and genotoxic stress [29-32]. Importantly, plant PARPs play an essential role in response to abiotic stresses. Transgenic *Arabidopsis* or oilseed rape (*Brassica napus*) plants with reduced PARP gene expression were more resistant to various abiotic stresses, including drought, high light and heat, partially attributed to a maintained energy homeostasis of reduced NAD+ and ATP consumption and alternation in plant hormone abscisic acid (ABA) levels in the transgenic plants [33,34]. The two *Arabidopsis* PARG genes, AtpARP1 and AtpARP2, which were likely derived from a tandem duplication event, locates next to each other on the same chromosome [23]. AtpARP1 (TEJ) was originally identified as a regulator of circadian rhythm and flowering in *Arabidopsis* [35]. Interestingly, the AtpARP2 gene was robustly induced by the treatments of MAMPs and various pathogens [36]. The plants carrying mutation in AtpARP1, but not AtpARP2, showed the elevated elf18 (a 18-amino-acid peptide of EF-Tu)-mediated seedling growth inhibition and phenylpropanoid pigment accumulation, suggesting a negative role of *Arabidopsis* PARG in certain plant immune responses [37]. Similar to AtPARP1, AtPARG1 also plays a role in plant drought, osmotic and oxidative stress tolerance [38]. In contrast to the extensive research efforts on PARPs/PARGs in animal systems, the biochemical activities and molecular actions of plant PARPs/PARGs remain poorly characterized.

To elucidate the signaling networks regulating immune gene activation, we developed a sensitive genetic screen with an ethyl methanesulfonate (EMS)-mutagenized population of *Arabidopsis* transgenic plants carrying a luciferase reporter gene under the control of the FRK1 promoter (pFRK1::LUC). The FRK1 (flg22-induced receptor-like kinase 1) gene is a specific and early immune responsive gene activated by multiple MAMPs [39,40]. A series of mutants with altered pFRK1::LUC activity upon flg22 treatment were identified and named as *Arabidopsis* genes governing immune gene expression (aggie). In this study, we isolated and characterized the *aggie2* mutant, which exhibited elevated immune gene expression upon multiple MAMP treatments. Map-based cloning coupled with next generation sequencing revealed that *Aggie2* encodes AtpARP1. Extensive biochemical analysis demonstrates that both AtpARP1 and AtpARP2 carry poly(ADP-ribose) polymerase activity, whereas AtpARP1, but not AtpARP2, possesses poly(ADP-ribose) glycohydrolase activity *in vivo* and *in vitro*. Significantly, the enzymatic activity of AtpARP2 is enhanced upon flg22 perception, suggesting the potential involvement of protein PARylation in MAMP-triggered immunity. The *aggie2* mutation (G450R) occurs at a highly conserved PARG residue which is essential for both *Arabidopsis* AtpARP1 and human HsPARG enzymatic activity. Consistent with the negative role of AtpARP1 in plant innate immunity, AtpARP1 and AtpARP2 positively regulate immune gene activation and plant resistance to virulent bacterial pathogen infection. Our results indicate that the reversible posttranslational PARylation process mediated by AtpARP5 and AtpARP6 plays a crucial role in mounting successful innate immune responses upon MAMP perception in *Arabidopsis*.

**Results**

The *aggie2* mutant displays enhanced immune gene expression

The *aggie2* mutant isolated from a genetic screen of the EMS-mutagenized pFRK1::LUC transgenic plants exhibits elevated FRK1 promoter activity upon flg22 treatment compared to its
Aggie2 encodes a putative poly(ADP-ribose) glycohydrolase

To isolate the causative mutation in aggie2, we crossed aggie2 (in the Col-0 accession background) with the Ler accession and mapped aggie2 to an 88 kilobase pair (kb) region between markers F20M17 and F22D22 on Chromosome 2 (Fig. 2A). We then performed Illumina whole genome sequencing of aggie2 and WT pFRK1::LUC transgenic plants. The comparative sequence analysis identified a G to A mutation at the position 13418 bp of At2g31870 within this 88 kb region. The mutation was further confirmed by Sanger sequencing of the genomic DNA of At2g31870. At2g31870 encodes AtPARG1 and the mutation in the aggie2 mutant causes an amino acid change of Glycine (G) at At2g31870.

To confirm that the G450R lesion in AtPARG1 is the causative mutation in aggie2, we complemented the aggie2 mutant with a construct carrying AtPARG1 cDNA fused with a FLAG epitope tag under the control of its native promoter (pAtPARG1::AtPARG1-FLAG). Two homozygous T3 transgenic lines, one line

Fig. 1. Elevated pFRK1::LUC expression and MAMP-triggered immune response in aggie2 mutant. (A) Luciferase activity from 10-day-old pFRK1::LUC (WT) and aggie2 seedlings treated with or without 10 nM flg22 for 12 hr. The photograph was taken with an EMCCD camera. The number below indicates quantified signal intensity shown as means ± se from 12 seedlings. (B) Time-course of pFRK1::LUC activity in response to 100 nM flg22 treatment. The data are shown as means ± se from at least 20 seedlings for each time point. (C) The pFRK1::LUC activity in response to different MAMPs. Ten-day-old seedlings were treated with 100 nM elf18, 50 μg/ml chitin, 1 μM LPS, or 500 ng/ml PGN for 12 hr. The data are shown as means ± se from at least 12 seedlings for each treatment. (D) The pFRK1::LUC activity triggered by different bacteria. Four-week-old soil-grown plants were hand-inoculated with different bacteria at the concentration of OD600 = 0.5. The data are shown as means ± se from at least 12 leaves for each treatment at 24 hr post-inoculation (hpi). (E) flg22-induced callose depositions in aggie2 mutant. Leaves of 6-week-old plants were infiltrated with 0.5 μM flg22 for 12 hr and callose deposits were detected by aniline blue staining and quantified by ImageJ software. (F) flg22-induced MAMP activation in aggie2 mutant. Seedlings were treated with 100 nM flg22 and collected at the indicated time points. The MAMP activation was detected with an α-pErk antibody (top panel) and the protein loading was indicated by Ponceau S staining for RUBISCO (Rbc) (bottom panel). (G) flg22-triggered ROS burst in aggie2 mutant. Leave discs from 4-week-old plants were treated with H2O or 100 nM flg22 over 30 min. The data are shown as means ± se from 20 leaf discs. (H) Endogenous MAMP-induced marker gene expression. Ten-day-old seedlings were treated with 100 nM flg22 and quantified by ImageJ software. The data are shown as means ± se from three biological repeats with Student’s t-test. * indicates p<0.05 and ** indicates p<0.01 when compared to WT. The above experiments were repeated 3 times with similar results.

doi:10.1371/journal.pgen.1004936.g001

parental line, pFRK1::LUC (WT) (Fig. 1A). The elevated luciferase activity in the aggie2 mutant was observed over a 40-hr time course period upon flg22 treatment (Fig. 1B). Notably, the aggie2 mutant did not display detectable enhanced FRK1 promoter activity in the absence of flg22 treatment, suggesting its specific regulation in plant defense. In addition to flg22, other MAMPs, including elf18, LPS, PGN and fungal chitin, also elicited the enhanced FRK1 promoter activity in the aggie2 mutant (Fig. 1C), indicating that Aggie2 functions as a convergent component downstream of multiple MAMP receptors. Consistently, the aggie2 mutant displayed the enhanced FRK1 promoter activity in response to the non-pathogenic bacterium Pseudomonas syringae pv. tomato (Pst) DC3000 hrcC defective in type III secretion of effectors, and a non-adaptive bacterium P. syringae pv. phaseolicola NPS3121 (Fig. 1D). The pathogenic bacterium Pst DC3000 failed to activate pFRK1::LUC, likely due to the suppression function of multiple effectors secreted from virulent bacterium [40]. Pathogen infection or purified MAMPs could induce callose deposits in leaves or cotyledons of Arabidopsis, which has emerged as an indicator of plant immune responses [41]. We compared callose deposits by aniline blue staining in WT and aggie2 mutant plants upon flg22 treatment. The aggie2 mutant deposited more callose than WT plants 12 hr after flg22 treatment, and the size of each callose deposit appeared bigger in the aggie2 mutant than that in WT plants (Fig. 1E).

We also detected MAPK activation and ROS production, two early events triggered by multiple MAMPs, in WT and aggie2 mutant. The flg22-induced MAPK activation detected by an α-pErk antibody did not show significant and reproducible difference in WT and aggie2 seedlings (Fig. 1F), suggesting that Aggie2 acts either independently or downstream of MAPK cascade. The flg22-induced ROS burst appeared to be similar in the aggie2 mutant compared to that in WT plants (Fig. 1G). We did not observe reproducible disease alternation in the aggie2 mutant compared to WT plants in response to Pst DC3000 infection either by hand-infiltration or spray-inoculation with various inoculums and conditions (S3A Fig.). Among 7 times of disease assays with Pst DC3000 hand-infiltration, we observed that aggie2 was slightly more resistant than WT plants for 4 times, whereas we did not see the significant difference between aggie2 and WT for other 3 times (S3A Fig). By contrast, the aggie2 mutant showed enhanced susceptibility to a necrotrophic fungus Botrytis cinerea compared to WT plants as evidenced by symptom development and lesion progression after infection (S3B Fig).

We further detected endogenous FRK1 expression in flg22-treated seedlings of WT and aggie2 mutant with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. The FRK1 expression was significantly elevated in the aggie2 mutant compared to that of WT pFRK1::LUC transgenic plants at both 30 min and 90 min after flg22 treatment (Fig. 1H). Similarly, the expression of several other early MAMP marker genes, including MYB15 and At2g17740 was also enhanced in the aggie2 mutant (Fig. 1H). Taken together, the results indicate that Aggie2 negatively regulates the expression of certain flg22-induced genes.
AtPARG1 and AtPARP2 carry poly(ADP-ribose) polymerase activity in vitro

AtPARG1 encodes a putative poly(ADP-ribose) glycohydrolase with a predicted activity to remove poly(ADP-ribose) polymers from the acceptor proteins catalyzed by poly(ADP-ribose) polymerases (PARPs). To elucidate the biochemical activity and function of AtPARGs, we first characterized the function of AtPARPs and established in vivo and in vitro protein PARylation assays. The Arabidopsis genome encodes three PARPs, AtPARP1, AtPARP2 and AtPARP3, with each consisting of a conserved PARP catalytic domain and a variable DNA binding domain (S2 Fig.). AtPARP1 and AtPARP3 carry zinc-finger domains for DNA binding, which is similar to human HsPARP-1, whereas AtPARP2 contains two SAP domains with putative DNA binding activity. The SAP domain was named after scaffold attachment factor A/B (SAF-A/B), apoptotic chromatin condensation inducer in the nucleus (Acinus) and protein inhibitors of activated STAT (PIAS), which all have DNA and chromatin binding ability and regulate chromatin structure and/or transcription [42]. Analysis of their tissue expression pattern suggests that AtPARP1 and AtPARP2 are expressed in leaves, whereas AtPARP3 is primarily expressed in developing seeds (S4B-S4C Fig.). Thus, we focused on AtPARP1 and AtPARP2 for the functional studies.

We first tested whether AtPARP1 and AtPARP2 carry poly(ADP-ribose) polymerase activity with recombinant proteins of AtPARP1 and AtPARP2 fused with Maltose Binding Protein (MBP). In the presence of activated DNA, both AtPARP1 and AtPARP2 could catalyze PARylation reaction by repeatedly transferring ADP-ribose groups from NAD+ to itself (auto-PARylation) as appeared a ladder-like smear with high-molecular-weight proteins in a Western blot using an α-PAR antibody which detects the PAR polymers of PARylated proteins (Fig. 3A). Apparently, AtPARP2 exhibited stronger in vitro enzymatic activity than AtPARP1 when detected by α-PAR antibody. The enzymatic activity of AtPARP1 and AtPARP2 was blocked by 3-AB, a competitive inhibitor of PARP (Fig. 3A). The activity of AtPARP2 is comparable with that of human HsPARP-1 (S5A Fig.). In addition, both AtPARP1 and AtPARP2 were able to transfer ADP-ribose from Biotin-NAD+ to itself and a relatively discrete band could be detected by horseradish peroxidase (HRP) conjugated streptavidin (Fig. 3B). The specificity of PARP activity was confirmed with 3-AB treatment, which dramatically reduced the activity. PARylation in Plant Immunity

With relatively low (C2-3) and another line with moderate (C4-1) expression of AtPARG1-FLAG, were chosen for complementation assays. Both lines restored WT level of pFRK1::LUC activity upon flg22 treatment either imaged with an EMCCD camera (Fig. 2C) or quantified by a luminometer (Fig. 2D), confirming that the enhanced FRK1 promoter activity in aggrie2 is caused by the mutation in AtPARG1. We also isolated T-DNA insertion line of AtPARG1, parg1-1 (SALK_147805) and parg1-2 (SALK_116088), and examined flg22-induced immune gene activation. Similar to the aggrie2 mutant, parg1-1 and parg1-2 displayed the elevated activation of FRK1, MYB15 and At2g17740 after flg22 treatment compared to WT Col-0 plants (Fig. 2E). PARP inhibitor disrupted MAMP-induced cell wall lignification [37]. We found that both parg1-1 and aggrie2 mutants showed the enhanced accumulation of lignin biosynthesis precursors, O-4-linked-coniferyl and sinapyl aldehydes, upon flg22 treatment by Wiesner staining (Fig. 2F). The complementation line C2-3 restored accumulation of these lignin biosynthesis precursors to the WT level (Fig. 2F). Consistent with a previous report [36], the transcript of AtPARG2, but not AtPARG1, was induced by flg22 treatment (S4A Fig.).

AtPARP1 and AtPARP2 carry poly(ADP-ribose) polymerase activity in vitro

AtPARG1 encodes a putative poly(ADP-ribose) glycohydrolase with a predicted activity to remove poly(ADP-ribose) polymers from the acceptor proteins catalyzed by poly(ADP-ribose) polymerases (PARPs). To elucidate the biochemical activity and function of AtPARGs, we first characterized the function of AtPARPs and established in vivo and in vitro protein PARylation assays. The Arabidopsis genome encodes three PARPs, AtPARP1, AtPARP2 and AtPARP3, with each consisting of a conserved PARP catalytic domain and a variable DNA binding domain (S2 Fig.). AtPARP1 and AtPARP3 carry zinc-finger domains for DNA binding, which is similar to human HsPARP-1, whereas AtPARP2 contains two SAP domains with putative DNA binding activity. The SAP domain was named after scaffold attachment factor A/B (SAF-A/B), apoptotic chromatin condensation inducer in the nucleus (Acinus) and protein inhibitors of activated STAT (PIAS), which all have DNA and chromatin binding ability and regulate chromatin structure and/or transcription [42]. Analysis of their tissue expression pattern suggests that AtPARP1 and AtPARP2 are expressed in leaves, whereas AtPARP3 is primarily expressed in developing seeds (S4B-S4C Fig.). Thus, we focused on AtPARP1 and AtPARP2 for the functional studies.

We first tested whether AtPARP1 and AtPARP2 carry poly(ADP-ribose) polymerase activity with recombinant proteins of AtPARP1 and AtPARP2 fused with Maltose Binding Protein (MBP). In the presence of activated DNA, both AtPARP1 and AtPARP2 could catalyze PARylation reaction by repeatedly transferring ADP-ribose groups from NAD+ to itself (auto-PARylation) as appeared a ladder-like smear with high-molecular-weight proteins in a Western blot using an α-PAR antibody which detects the PAR polymers of PARylated proteins (Fig. 3A). Apparently, AtPARP2 exhibited stronger in vitro enzymatic activity than AtPARP1 when detected by α-PAR antibody. The enzymatic activity of AtPARP1 and AtPARP2 was blocked by 3-AB, a competitive inhibitor of PARP (Fig. 3A). The activity of AtPARP2 is comparable with that of human HsPARP-1 (S5A Fig.). In addition, both AtPARP1 and AtPARP2 were able to transfer ADP-ribose from Biotin-NAD+ to itself and a relatively discrete band could be detected by horseradish peroxidase (HRP) conjugated streptavidin (Fig. 3B). The specificity of PARP activity was confirmed with 3-AB treatment, which dramatically reduced the activity. PARylation in Plant Immunity

With relatively low (C2-3) and another line with moderate (C4-1) expression of AtPARG1-FLAG, were chosen for complementation assays. Both lines restored WT level of pFRK1::LUC activity upon flg22 treatment either imaged with an EMCCD camera (Fig. 2C) or quantified by a luminometer (Fig. 2D), confirming that the enhanced FRK1 promoter activity in aggrie2 is caused by the mutation in AtPARG1. We also isolated T-DNA insertion line of AtPARG1, parg1-1 (SALK_147805) and parg1-2 (SALK_116088), and examined flg22-induced immune gene activation. Similar to the aggrie2 mutant, parg1-1 and parg1-2 displayed the elevated activation of FRK1, MYB15 and At2g17740 after flg22 treatment compared to WT Col-0 plants (Fig. 2E). PARP inhibitor disrupted MAMP-induced cell wall lignification [37]. We found that both parg1-1 and aggrie2 mutants showed the enhanced accumulation of lignin biosynthesis precursors, O-4-linked-coniferyl and sinapyl aldehydes, upon flg22 treatment by Wiesner staining (Fig. 2F). The complementation line C2-3 restored accumulation of these lignin biosynthesis precursors to the WT level (Fig. 2F). Consistent with a previous report [36], the transcript of AtPARG2, but not AtPARG1, was induced by flg22 treatment (S4A Fig.).
We first developed an in vivo PARylation assay with transiently expressed AtPARP2 tagged with an HA epitope at the C-terminus in Arabidopsis protoplasts. After feeding the cells with [32P]-NAD, the AtPARP2 proteins were immunoprecipitated with an α-HA antibody and separated in SDS-PAGE. A band corresponding to the predicted molecular weight of AtPARP2 was observed with autoradiograph, indicating in vivo AtPARP2 activity (Fig. 3E). This band is specific to AtPARP2 since it was absent in the vector control transfected cells. Strikingly, the flg22 treatment enhanced AtPARP2 in vivo PARylation activity as detected by increased band intensity with autoradiograph. Apparently, the flg22-mediated enhancement of AtPARP2 activity was not due to the increase of protein expression after treatment (Fig. 3E). The data demonstrate that AtPARP2 possesses poly(ADP-ribose) polymerase activity in vivo and AtPARP2-mediated protein PARylation is regulated by flg22 signaling. We further examined AtPARP2-GFP localization with Agrobacterium-mediated Nicotiana benthamiana transient assay. A strong fluorescence signal from AtPARP2-GFP was exclusively detected in the nucleus (Fig. 3F), which is consistent with its potential role in DNA repair, chromatin modulation and transcriptional regulation.

AtPARG1, but not AtPARG2, is a functional PARG enzyme

We next tested whether AtPARG1 and AtPARG2 possess poly(ADP-ribose) glycohydrolase activity (Fig. 4A). We isolated and purified AtPARG1 and AtPARG2 proteins fused with glutathione S-transferase (GST) expressed from E. coli, and established an in vitro PARG assays to examine whether AtPARGs could remove PAR from auto-PARylated AtPARP2 detected in a Western blot with an α-PAR antibody, suggesting the PARG activity of AtPARG1 towards AtPARP2. However, AtPARG2 appeared to be inactive towards auto-PARylated AtPARP2 in this assay (Fig. 4B). Similarly, AtPARG1, but not AtPARG2, could remove PAR polymers from auto-ADP-ribosylated AtPARP2 as detected with [32P]-NAD autoradiograph (Fig. 4C). We further examined whether AtPARG2 may possess PARG activity specifically towards AtPARP1 but not AtPARP2. As shown in Fig. 4C, AtPARG2 did not remove PAR polymers from auto-ADP-ribosylated AtPARP1. The 6xHistidine (His6)-tagged AtPARG2 also did not display in vitro enzymatic activity (S5B Fig.). Similar to the above assays using in vitro expressed AtPARG1 proteins (Fig. 4B & 4C), the immunoprecipitated AtPARG1 expressed in Arabidopsis protoplasts almost completely removed PAR polymers from in vivo PARylated AtPARP2 (Fig. 4D). Furthermore, AtPARG1 was able to remove PAR polymers from auto-PARylated human HsPARG-PARylated AtPARP1 (S5C Fig.). Similarly, human HsPARG was also able to remove PAR polymers from AtPARG2 (S5D Fig.), suggesting the functional conservation of human and Arabidopsis PARGs.
tagged PARG1, aggie2 or PARG2 proteins in the presence of \(^{32}\text{P}-\text{NAD}\) PARylated and further subjected for part of top panel). MBP-AtPARP1 or AtPARP2 proteins were auto-PARylated and further subjected for in vitro PARP assay using GST-tagged AtPARP1, aggie2 or AtPARP2 proteins. The PARylated proteins were detected with an \(\alpha\)-PAR antibody. MBP-AtPARP1 or AtPARP2 proteins were auto-PARylated and further subjected for in vitro PARP assays using GST-tagged PAR1, aggie2 or PARG2 proteins in the presence of \(^{32}\text{P}-\text{NAD}\). The PARylated proteins were detected with autograph (top panel) and the protein inputs are shown with CBB staining (bottom panel). (C) The AtPARP1 in vitro activity detected with \(^{32}\text{P}-\text{NAD}\) PARylase. AtPARP1, but not AtPARP2 or aggie2, possesses in vitro PARP activity towards auto-PARylated AtPARP2 proteins detected by \(\alpha\)-PAR antibody. MBP-AtPARP2 proteins were auto-PARylated and further subjected for in vitro PARP assay using GST-tagged AtPARP1, aggie2 or AtPARP2 proteins. The PARylated proteins were detected with an \(\alpha\)-PAR Western blot (top panel) and the protein inputs are shown with CBB staining (bottom panel). (D) Protoplast-expressed AtPAR1 possesses PARP activity towards in vitro auto-PARylated AtPARP2 proteins. Arabidopsis protoplasts were co-transfected with AtPAR1-FLAG or vector control and treated with or without 100 mM flg22 for 15 min. PAR1 proteins were immunoprecipitated with \(\alpha\)-FLAG antibody and subjected for in vitro PARP assay with in vitro auto-PARylated MBP-AtPARP2 proteins. The PARylated proteins were detected in an \(\alpha\)-PAR Western blot (top panel), MBP-AtPARP1 or AtPARP2 proteins were auto-PARylated and further subjected for in vitro PARP assays using GST-tagged PAR1, aggie2 or PARG2 proteins in the presence of \(^{32}\text{P}-\text{NAD}\). The PARylated proteins were detected with autograph (top panel) and the protein inputs are shown with CBB staining (bottom panel). (D) Protoplast-expressed AtPAR1 possesses PARP activity towards in vitro auto-PARylated AtPARP2 proteins. Arabidopsis protoplasts were co-transfected with AtPAR1-FLAG or vector control and treated with or without 100 mM flg22 for 15 min. PAR1 proteins were immunoprecipitated with \(\alpha\)-FLAG antibody and subjected for in vitro PARP assay with in vitro auto-PARylated MBP-AtPARP2 proteins. The PARylated proteins were detected in an \(\alpha\)-PAR Western blot (top panel), MBP-AtPARP1 or AtPARP2 proteins were auto-PARylated and further subjected for in vitro PARP assays using GST-tagged PAR1, aggie2 or PARG2 proteins in the presence of \(^{32}\text{P}-\text{NAD}\). The PARylated proteins were detected with autograph (top panel) and the protein inputs are shown with CBB staining (bottom panel). (E) AtPAR1, but not AtPAR2, has in vivo PAR glycohydrolase activity. AtPAR2-FLAG was co-expressed with vector control, AtPAR1-HA or AtPAR2-HA in protoplasts and, the protein inputs were fed with \(^{32}\text{P}-\text{NAD}\). The PARylated proteins were detected with autograph after immunoprecipitation with \(\alpha\)-FLAG antibody (top panel). The PARP and PARG protein expression was detected with Western blot (middle panels) and the protein loading is shown with Ponceau S staining (bottom panel). (F) Subcellular localization of AtPARG1 and AtPARG2 in protoplasts. AtPARG1-GFP or AtPARG2-GFP was transiently expressed in protoplasts and the images were taken 12 hr after transfection using a confocal microscope. NLS-RFP was co-transfected for nuclear localization control. The above experiments were repeated 3 times with similar results.

doi:10.1371/journal.pgen.1004936.g004

The aggie2 mutation occurs at a conserved and essential PARP residue

We further addressed whether the aggie2 (G450R) mutation affected its PARP activity. Significantly, the aggie2 (G450R) mutant of AtPARG1 completely abolished its enzymatic activity detected by either \(\alpha\)-PAR antibody (Fig. 4B) or \(^{32}\text{P}-\text{NAD}\) autoradiography-based assay (Fig. 4C). Notably, the G450 in AtPARG1 is highly conserved among PARPs of different species (Fig. 2B). Interestingly, the corresponding mutation in human HsPARG (G867R) also abolished its activity towards HsPARP-1 and AtPARG2, suggesting the essential role of this highly conserved residue in different PARPs and potentially divergent evolution.

AtPARPs positively regulate plant immunity

We tested the involvement of AtPARPs in plant innate immunity and immune gene activation. Because of the potential functional redundancy of AtPARP1 and AtPARP2 [30,31], we
performed disease assay and analyzed defense gene expression in atparp1 atparp2 (atparp1/2) double mutant. The atparp1/2 mutant plants were more susceptible to virulent P. syringae pv. maculicola ES4326 (Psm) infection compared to WT plants as indicated by more than 10 fold increase of bacterial growth in the atparp1/2 mutant (Fig 6A). The disease symptom development was more pronounced in the atparp1/2 mutant than WT plants (Fig 6A). Similarly, the atparp1/2 mutant plants showed the enhanced susceptibility with bacterial growth and symptom development to the infections by Pst DC3000 and a less virulent bacterium Pst DC3000ΔavrPtoavrPtoB (Fig 6B & S7 Fig.). In addition, the atparp1/2 mutant plants showed the reduced induction of MAMP marker genes, including FRK1 and At2g17740, compared to WT plants at 90 min after flg22 treatment (Fig 6C). Together, these data indicate that AtPARP1 and AtPARP2 are positive regulators in plant immunity and defense gene activation to bacterial infections.

Discussion
Protein PARylation mediated by PARPs and PARGs is an important, but less understood posttranslational modification process implicated in the regulation of diverse cellular processes and physiological responses [26]. In this study, an unbiased genetic screen revealed that Arabidopsis AtPARG1 plays an important role in regulating immune gene expression upon pathogen infection. We established and performed extensive in vitro and in vivo biochemical assays of PARP and PARG enzymatic activities. We have shown for the first time that Arabidopsis AtPARP1 and AtPARP2 are able to transfer ADP-ribose moieties from NAD⁺ to itself and acceptor proteins in vitro and in vivo. Thus, they are bona fide poly(ADP-ribose) polymerases. Interestingly, in contrast to their mammalian counterparts, AtPARP2 is more enzymatically active than AtPARP1. Significantly, MAMP perception promotes substantial enhancement of AtPARP2 enzymatic activity in vivo, reconciling the biological importance of PARPs/PARGs in regulating immune gene expression. AtPARG1, but not AtPARG2, is able to remove PAR polymers from PARYlated proteins in vivo and in vitro and it is a bona fide poly(ADP-ribose) glycohydrolase. The Arabidopsis parp1 (agg2) mutant plants exhibited elevated expression of several MAMP-induced genes and callose deposition. Conversely, the Arabidopsis atparp1/2 mutant showed reduced expression of MAMP-induced genes and enhanced susceptibility to virulent Pseudomonas infections. Thus, the data suggest that protein PARylation positively regulates certain aspects of plant immune responses. Notably, the viability and normal growth of Arabidopsis parp and parg null mutants represent a unique opportunity to study protein PARylation regulatory mechanisms in diverse biological processes at the whole organismal level.

Our results lend support to a previous study that treatment of pharmacological inhibitor of PARPs, 3-AB, disrupted elf18- and/or flg22-induced callose and lignin deposition, pigment accumulation and phenylalanine ammonia lyase activity [37]. However, the flg22-induced defense genes (FRK1 and WRK129) were not affected by 3-AB treatment [37]. Our study with Arabidopsis parp and parp genetic mutants revealed a previously unrecognized function of protein PARylation in regulating immune gene expression upon pathogen infection. This is consistent with the general role of human PARPs and PARG in transcriptional regulation and chromatin modification [43,45] and further substantiates the hypothesis that plant PARPs could ameliorate the cellular stresses caused by antimicrobial defenses (e.g. the effects of elevated ROS levels) [23]. Interestingly, ADP-ribosylation has also been exploited by pathogens as a means to quell plant immunity. Two Pseudomonas syringae effectors, HopU1 and HopF2, mono-ADP-ribosylate RNA-binding protein GRP7 and MAPK kinase MKK5 respectively, and interfere with their activities in plant defense transcription regulation and signaling [46,47].

Unlike mammals and most other animals that encode a single PARG gene, the Arabidopsis genome encodes two adjacent PARG genes, AtPARG1 and AtPARG2, as well as a pseudogene At2g31860. Surprisingly, only AtPARG1, but not AtPARG2, possesses detectable poly(ADP-ribose) glycohydrolase activity in vitro and in vivo with our extensive biochemical assays. Sequence analysis identified a polymorphism in the conserved PARG signature motif “GGG-X–QEE”, where the third G is replaced with an L in AtPARG2. The PARG signature motif is absolutely required for its enzymatic activity as mutations at this motif in AtPARG1 completely abolished its activity. However, creation of the conserved signature motif in AtPARG2 was unable to gain its PARG activity suggesting that other polymorphisms in AtPARG2 are also responsible for its lack of enzymatic activity. Consistent with our biochemical assays, the PAR polymer concentration was much higher in atparp1 mutant than that in WT plants. A similar conclusion was reached on tey mutant, which carries a G262E mutation in the invariable signature motif of AtPARG1 [35]. The atparp1, but not atparp2 mutant, affected elf18-induced seedling growth inhibition and pigment formation, and sensitivity to DNA-damaging agent [37]. Interestingly, AtPARG2 is substantially induced in multiple plant-pathogen interactions [36] and it is required for plant resistance to B. cinerea infections [37]. Thus, despite of lacking detectable enzymatic activity, AtPARG2 may still play certain role in plant immunity. It is possible that AtPARG2 may regulate AtPARG1 activity. It is also possible that
AtPARG2 has evolved novel functions in plant immune responses. Several other plant species, including rice, poplar, tomato and maize, are also predicted to encode multiple PARGs [23] (S6 Fig.). Unlike Arabidopsis PARGs, different PARG members in other species have invariant signature motif. For example, all three PARGs in poplar contain GGG-X-Y-QEE signature motif (S6 Fig.). However, a few other species such as Extrema salugiunum, Capella rubella, Phaseolus vulgaris, Oskopeura dioica, and Xenopus laevius, contain PARGs with an AtPARG2-like signature GGL-X-Y-QEE. It remains unknown how many PARPs are enzymatic active in the species with multiple PARGs.

Although there are 17 PARPs in mammals, the parp-1/tharp-2 double mutant mice are not viable and die at the onset of gastrulation, suggesting the essential role of protein PARylation during early embryogenesis [48]. The lethality of parp-1/tharp-2 double mutant mice might be due to genomic instability. However, Arabidopsis atparp1/2 double mutant is largely morphologically similar with WT plants and does not display any obvious growth defects. Although Arabidopsis atparp1/2 double mutant was hypersensitive to genotoxic stress, they did not have significant changes in telomere length nor end-to-end chromosome fusions [30]. Albeit mainly expressed in developing seeds, AtPARP3 may have redundant functions with AtPARP1 and AtPARP2 in maintaining genome stability. It remains interesting whether atparp1/2/3 triple mutant will exert abnormal plant growth and development. Consistent with the essential function of PARylation during embryogenesis, PARG-deficient mice and Drosophila are embryonic lethal which is probably due to the accumulation of PAR polymers and uncontrolled PAR-dependent signaling [49,50]. The normal plant growth phenotype of atparp1 mutant might be due to the redundant function of AtPARP2. However, our extensive biochemical analysis indicates that AtPARG1, but not AtPARG2, accounts for most of PARG enzymatic activity. As AtPARG1 and AtPARG2 reside next to each other on the same chromosome, it is challenging to generate the double mutant. It remains possible that other PAR-degrading enzymes with distinct sequences exist in Arabidopsis. In vertebrate, ADP-ribosyl hydrolyase 3 (ARH3), a structurally distinct enzyme from PARG, could also degrade PAR polymers associated with the mitochondrial matrix [26].

We observed that AtPARP2 activity was rapidly and substantially stimulated by flg22 treatment. In line with this observation, it has been shown that bacterial infections induced the increase of PAR polymers in Arabidopsis [37]. It is well established that damaged DNA stimulates PARP activity. Recent studies have shown that pathogen treatments induce DNA damage [51,52], which could potentially serve as a trigger to activate PARP. Treatments with virulent or avirulent Pst strains for hours could induce DNA damage in Arabidopsis as detected by abundance of histone γ-H2AX, a sensitive indicator of DNA double-strand breaks or by DNA comet assays [51]. Prolonged pathogen treatment is often accompanied with the elevated accumulation of plant defense hormone salicylic acid (SA). It has also been shown that SA can also trigger DNA damage in the absence of a genotoxic agent [51]. However, treatments of flg22 or elf18 did not induce detectable DNA damage [51]. In addition, flg22-mediated stimulation of ApARPA2 activity occurs rather rapidly and within 30 min after treatment. Apparently, flg22 signaling could directly activate ApARPA2. It is well known that human HsPARP-1 is regulated by different posttranslational modification processes, such as phosphorylation, ubiquitination, SUMOylation and cleavage [22]. HsPARP-1 could be activated by phosphorylated MAPK ERK2 in a broken DNA-independent manner, thereby enhancing ERK-induced Elk1 phosphorylation, core histone acetylation, and transcription of the Elk1-target genes [54]. MAPK cascade plays a central role functioning downstream of multiple MAMP receptors. It will be interesting to test whether flg22-activated MAPKs directly modulate PARP and/or PARG activities.

Our genetic and biochemical analyses revealed that PARP/PARG-mediated PAR dynamics regulates immune gene expression in Arabidopsis. Mammalian PARPs/PARGs regulate gene expression through a variety of mechanisms including modulating chromatin, functioning as transcriptional co-regulators and mediating DNA methylation [53]. PARylation of histone lysine demethylase KDM5B maintains histone H3 lysine 4 trimethyl (H3K4me3), a histone mark associated with active promoters, by inhibiting KDM5B demethylase activity and interactions with chromatin. In addition, HsPARP-1 is able to promote exclusion of H1 and opening of promoter chromatin, which collectively lead to a permissive chromatin environment that allows loading of the RNAII machinery [45]. HsPARP is also able to promote the formation of a chromatin environment suitable for retinoic acid receptor (RAR)-mediated transcription by removing PAR polymer from PARylated H3K9 demethylase KDM4D/JMJ2D thereby activating KDM4D/JMJ2D to inhibit H3K9me2, a histone mark associated with transcriptional repression [43]. Arabidopsis PARGs and PARGs are localized in the nucleus, and AtPARP2 could PARylate Histone H1. It is plausible to speculate that similar modes of action of protein PARylation-mediated transcriptional regulation exist in plants. Future identification of PARP/PARG targets (promoters and proteins) and PAR-associated proteins, especially during plant immune responses, will elucidate how protein PARylation modulates plant immune gene expression.

**Materials and Methods**

**Plant and pathogen materials and growth conditions**

Arabidopsis accession Col-0, pFRK1::LUC transgenic plants, aggie2 mutant, atparp1-1 (SALK_147805), atparp1-2 (SALK_16088), atparp2 (GABI-Kat 072B04), atparp1/atparp2 (GABI-Kat 692A05/SALK_640400), pPARG1::PARG1-FLAG transgenic plants were grown in soil (Metro Mix 366) at 23°C, 60% humidity and 75 μE m⁻² s⁻¹ light with a 12-hr light/12-hr dark photoperiod. Four-week-old plants were used for protoplast isolation and transient expression assays according to the standard procedure [55]. Seedlings were germinated on 1/2 MS containing 1% sucrose, 0.1% glutamic acid, 1.5% agar) with appropriate antibiotics. Bacterial colony forming units (cfu) were counted 2 days after incubation at 28°C. For transgenic Arabidopsis atparp1/atparp2, aggie2 mutants, parp-1parp-2 (NPS3121 strains were cultured overnight at 28°C with appropriate antibiotics. Bacteria were harvested by centrifugation, washed, and adjusted to the desired density with 10 mM MgCl₂. Leaves of 4-week-old plants were hand-infiltrated with bacterial suspension using a 1-ml needleless syringe and collected at the indicated time for luciferase activity or bacterial growth assays. To measure bacterial growth, two leaf discs were ground in 100 μl H₂O and serial dilutions were plated on TSA medium (1% Bacto tryptone, 1% sucrose, 0.1% glutamic acid, 1.5% agar) with appropriate antibiotics. Bacterial colony forming units (cfu) were counted 2 days after incubation at 28°C. Each data point is shown as triplicates. Botrytis cinerea strain BO5 was cultured on Potato Dextrose Agar (Difco) and incubated at room temperature.
Conidia were re-suspended in distilled water and spore concentration was adjusted to 2.5 × 10^7 spores/ml. Gelatin (0.5%) was added to conidial suspension before inoculation. Leaves of six-week-old plants were drop-inoculated with B. cinerea at the concentration of 2.5 × 10^7 spores/ml. Lesion size was measured 2 days post-inoculation.

**Mutant screening, map-based cloning and next generation sequencing**

The **pFRK1::LUC** construct in a binary vector was transformed into *Arabidopsis* Col-0 plants. The homozygous transgenic plants with flg22-inducible **pFRK1::LUC** were selected for mutagenesis. The seeds were mutagenized with 0.1% ethane methyl sulfonate (EMS). Approximately 6,000 M2 seedlings were screened for their responsiveness to flg22 treatment. The seedlings were germinated in liquid ½ MS medium for 14 days, and then transferred for water for overnight and treated with 10 nM flg22. After 12 hr flg22 treatment, the individual seedlings were transferred to a 96-well plate, sprayed with 0.2 mM lincomycin and kept in dark for 20 min. The bioluminescence from induced **pFRK1::LUC** expression was recorded by a luminometer (Perkin Elmer, 2030 Multilabel Reader, Victor X3). The candidate mutants with altered flg22 responsiveness were recovered on ½ MS plate for 10 days, and then transferred to soil for seeds.

The aggie2 mutant was crossed with *Arabidopsis Ler* accession, and an F2 population was used for map-based cloning. Mapping with 270 F2 plants with aggie2 mutant phenotype placed the causal mutation in an 88 kb region between marker F20F17 and F22D22 on chromosome 2. The aggie2 genomic DNA was sequenced with the 100 nt paired-end sequencing on an Illumina HiSeq 2000 platform at Texas AgriLife Genomics and Bioinformatics Service (TAGS) (College Station, TX, USA). Ten-fold genome coverage was obtained with 11M reads. The Illumina reads were analyzed using CLC Genomics Workbench 6.0.1 software. By mapping to Col-0 genomic sequence (TAIR10 release), SNPs were identified as candidates of aggie2 mutation. In the aforementioned 88 kb region, a G to A mutation at the position of 1346 nt of At2g31870 was identified with 100% frequency. The mutation was confirmed by Sanger sequencing of aggie2 genomic DNA.

**Plasmid constructs for protoplasts and transgenic plants**

The AtPARP1, AtPARP2, AtPARG1, AtPARG2 and Histone H1.1 (At1g06760) genes were amplified from *Arabidopsis* Col-0 cDNA and cloned into a plant transient expression vector (pHBT vector) with an HA, FLAG or GFP epitope tag at the C-terminus via restriction sites NcoI or BamHI and StuI respectively. The two flag-NOS terminator fragment was released from pHBT-AtPARP2-HA. After 12 hr incubation, the protoplasts were treated with 10 nM flg22 for 30 min and fed with 1 μCi 32P-NAD^+. After 1 hr, the protoplasts were lysed in IP buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 1 × protease inhibitor, 1 μm DTT, 2 mM NaF and 2 mM Na3VO4) and the AtPARP2-Flag proteins were immunoprecipitated with α-HA antibody (Roche, USA) and protein-G-agarose (Roche, USA) in a shaker for 3 hr at 4°C. In vitro PARylated proteins enriched on the beads were then separated in 10% SDS-PAGE and visualized by autoradiography.

For in vivo PAR assays, 500 μl *Arabidopsis* protoplasts at the concentration of 2 × 10^6/ml were transfected with 100 μg of plasmid DNA of pHB-T-AtPARP2-HA. After 12 hr incubation, the protoplasts were treated with 100 nM flg22 for 30 min and fed with 1 μCi 32P-NAD^+ for 1 hr. The protoplasts were then lysed in IP buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 1× protease inhibitor, 1 μM DTT, 2 mM NaF and 2 mM Na3VO4) and the AtPARP2-Flag proteins were immunoprecipitated with α-HA antibody (Roche, USA) and protein-G-agarose (Roche, USA) in a shaker for 3 hr at 4°C. In vivo PARylated proteins enriched on the beads were then separated in 10% SDS-PAGE and visualized by autoradiography.

For in vivo PAR assay, AtPARG1-HA or AtPARG2-HA plasmid DNA was co-transfected with AtPARP2-Flag plasmid DNA into protoplasts, and expressed for 12 hr. The protoplasts were then incubated in 25 mM PAR assay, 25 mCi 32P-NAD^+ for 1 hr. The protoplasts were then lysed in IP buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 1× protease inhibitor, 1 μM DTT, 2 mM NaF and 2 mM Na3VO4) and the AtPARP2-Flag proteins were immunoprecipitated with α-HA antibody (Roche, USA) and protein-G-agarose (Roche, USA) in a shaker for 3 hr at 4°C. In vivo PARylated proteins enriched on the beads were then separated in 10% SDS-PAGE and visualized by autoradiography.

Detection of PAR polymers from protein extract of nuclei

The 12-day old seedlings grown on ½ MS plates were harvested and ground into fine powder in liquid nitrogen. Isolation of nuclei with Honda buffer was performed according to published procedure [37]. Nuclear proteins were released in lysis buffer with 1% SDS and spotted on nitrocellulose membrane. The protein loaded on the membrane was normalized by using α-Histone H3 vector was transformed into aggie2 via Agrobacterium-mediated transformation.

The primers for cloning and point mutations were listed in the S1 Table.

**In vitro and in vivo PARP and PARG assays**

Expression and purification of GST, His6 and MBP fusion proteins were performed according to the manufacturer’s manuals. For in vitro auto-PARYlation reaction, 1.2 μg of MBP-AtPARP2 or MBP-AtPARP1 proteins were incubated in a 20 μl reaction with 1 × PAR reaction buffer (50 mM Tris-HCl, pH8.0, 50 mM NaCl) with 0.2 mM NAD^+ and 1 × activated DNA (Trevigen, USA). To inhibit PAR reaction, 2.5 mM PARP inhibitor, 3-Aminobenzamide (3-AB, Sigma, USA) was added to the reaction. The reactions were kept at room temperature for 30 min and stopped by adding SDS loading buffer. To detect PARG activity, about 1.0 μg of purified GST, GST-AtPARG1 or GST-AtPARG2 proteins together with 2.5 μM 3-AB were added to auto-PARylated AtPARP2 proteins derived from the above PAR reactions and incubated at room temperature for another 30 min. PARylated proteins were separated in 7.5% SDS-PAGE and detected with an α-PAR polyclonal antibody (Trevigen, USA). For Biotin NAD^+ PAR assay, 25 μM Biotin-NAD^+ (Trevigen, USA) was added to replace NAD^+ in the reaction described above. The PAR polymer formation was detected by Streptavidin-HRP (Pierce, USA). For in vitro 32P-NAD^+-mediated PAR assays, 1.0 μg of MBP-AtPARP2 or MBP-AtPARP1 proteins were incubated in a 20 μl reaction in the buffer containing 50 mM Tris-HCl, pH8.0, 4 mM MgCl2, 300 mM NaCl, 1 mM DTT, 0.1 μg/ml BSA, 1 × activated DNA, 1 μCi 32P-NAD^+ (Perkin Elmer, USA) and 100 nM cold NAD^+ for 30 min at room temperature. For Histone PARylation assays, 2.0 μg of MBP-H1.1 or MBP-H1.3 proteins were added in the above reactions. The radiolabeled proteins were separated in SDS-PAGE and visualized by autoradiography.
ferred to 2 ml H2O in a 6-well plate to recover for 1 day, and then treated with 100 nm flg22 for 30 or 90 min. RNA was extracted using TRizol reagent (Life Technologies, USA) and quantified with NanoDrop. The RNA was treated with RNase-free DNase I (Promega, USA) for 30 min at 37°C, and then reverse transcribed with M-MuLV Reverse Transcriptase (NEB, USA). Real-time RT-PCR was carried out using iTaq Universal SYBR Green Mix (Bio-Rad, USA) on 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The primers used to detect specific transcript by real-time RT-PCR are listed in S2 Table.

Callose deposition
Leaves of six-week-old plants grown in soil were hand-inoculated with 0.5 μM flg22 or H2O for 12 hr. The leaves were then transferred into FAA solution (10% formaldehyde, 5% acetic acid and 50% ethanol) for 12 hr, de-stained in 95% ethanol for 6 hr, washed twice with ddH2O, and incubated in 0.01% aniline blue solution (150 mM KH2PO4, pH 9.5) for 1 hr. The callose deposits were visualized with a fluorescence microscope. Callose deposits were counted using ImageJ 1.43U software (http://rsb.info.nih.gov/ij/).

Lignin deposition
Leaves of six-week-old plants grown in soil were surface-sterilized by 70% ethanol, rinsed with H2O and incubated with 100 nM flg22 or H2O for 12 hr. The leaves were then de-stained in 95% ethanol with 2% chloroform for 12 hr and 95% ethanol in 2% phosphoric acid (20% ethanol, 20% HCl) for 5 min. The images were scanned by HP officejet Pro 8600 Premium.

MAPK assay
Ten-day-old seedlings germinated on ½ MS plate were transferred to 2 ml H2O in a 6-well plate to recover for 1 day, and then treated with 100 nM flg22 for 5, 15 or 45 min. The seedlings were grinded in IP buffer. The cleared lysate was mixed with SDS sample buffer and loaded onto 12.5% SDS-PAGE. Activated MAPKs were detected with α-pErk1/2 antibody (Cell Signalling, USA).

ROS analyses
ROS burst was determined by a luminol-based assay. At least 10 leaves of four-week-old Arabidopsis plants for each genotype were excised into leaf discs of 0.25 cm2, followed by an overnight incubation in 96-well plate with 100 μl of H2O to eliminate the wounding effect. H2O was replaced by 100 μM horseradish peroxidase (Sigma, USA) supplemented with or without 100 nM flg22. The measurement was conducted immediately after adding the solution with a luminometer (Perkin Elmer, 2030 Multilabel Reader, Victor X3), with a 1.5 min interval reading time for a period of 30 min. The measurement values for ROS production from 20 leaf discs per treatment were indicated as means of RLU (Relative Light Units).

GFP localization assay
Arabidopsis protoplasts were transfected with various GFP-tagged pHBT constructs as indicated in the figures. Fluorescence signals in the protoplasts were visualized under a confocal microscope 12 hr after transfection. To construct 35S::AtPARP2-GFP binary plasmid for Agrobacterium-mediated transient assay, the NcoI-PstI fragment containing AtPARP2-GFP was released from pHBT-35S::AtPARP2-GFP and ligated into pCB302 binary vector. For tobacco transient expression, Agrobacterium tumefaciens strain GV3101 containing pCB302-35S::AtPARP2-GFP was cultured at 28°C for 18 hr. Bacteria were harvested by centrifugation at a speed of 3500 rpm and resuspended with infiltration buffer (10 mM MES pH = 5.7, 10 mM MgCl2, 200 μM acetosyringone). Cell solution at OD600 = 0.75 was used to infiltrate 3-week-old Nicotiana benthamiana leaves. Fluorescence signals were detected 2 days post-infiltration. Fluorescence images were taken with Nikon-AI confocal laser microscope systems and images were processed using NIS-Elements Microscope Imaging Software. The excitation lines for imaging GFP, RFP and chloroplast were 488, 561 and 640 nm, respectively.

Supporting Information
S1 Fig. PARP- and PARG-mediated posttranslational PARylation in cellular stress responses. Extrinsic and intrinsic stress signals activate PARP which transfers ADP-ribose moiety from NAD+ to acceptor proteins resulting in the formation of linear or branched poly(ADP-ribose) (PAR) polymers. PARG could also be activated by different stresses and remove PAR polymers from acceptor proteins. Nucleoside diphosphate linked to some moiety-X (NUDX) then cleaves free ADP-ribose into AMP (adenosine monophosphate) and ribose-5-phosphate. (TIF)

S2 Fig. Domain organization of PARPs and PARGs. (A) Domain organization of human HsPARPs and Arabidopsis AtPARPs; ZF: PARP-like zinc-finger1 domain; ZFII: PARP-like zinc-finger2 domain; ZF(PADR): zinc-binding domain 3; BRCT: BRCA1 carboxy-terminal domain for protein–protein and protein–DNA break binding domain; WGR: Trp-Gly-Arg in single letter code for putative PARP nucleic acid binding domain; PRD: PARP regulatory domain; PARP: PARP catalytic domain; SAP: SAF-A/B, Acinus and PIAS motif for putative DNA/RNA binding domain; (B) Domain structure of human HsPARP, Rat RnPARG and Arabidopsis AtPARGs. A-domain: N-terminal regulatory and targeting domain; MTS: mitochondrial targeting sequence; Macrodomain fold: core catalytic domain. The number under each domain indicates the position of amino acid in the protein. (TIF)

S3 Fig. Disease assays in aggie2. (A) The aggie2 mutant response to Pst DC3000 infection. WT and aggie2 mutant plants were hand-inoculated with Pst DC3000 at OD600 = 5 × 10−1, and the bacterial counting was performed 3 days post-inoculation (dpi). The data are shown as mean ± se from three independent repeats. We performed 7 times of disease assays, and observed that aggie2 was more resistant than WT plants for 4 times, and there is no difference between aggie2 and WT for other 3 times. The representative bacterial counting with difference (left) or without difference (right) is shown. (B) The aggie2 mutant is more susceptible to B. cinerea infection. Leaves of six-week-old plants were drop-inoculated with B. cinerea at the concentration of 2.5 ×
10^5 spores/mL. Lesion size was measured 2 days post-inoculation. The data are shown as mean ± se from 20 infected leaves.

(TIF)

S4 Fig. Expression pattern of AtPARG1, AtPARG2, AtPARP1, AtpARP2 and AtpARP3. (A) Response of AtPARG1 and AtpARP2 transcript level to flg22 treatment. The 12-day-old seedlings were treated with 100nM flg22 for qRT-PCR analysis. (B) The transcription levels of AtPARP1, AtpARP2 and AtpARP3 in 6 primary organs (Rs, root; St, stem; Lf, leaf; Inf, inflorescence; Sq, silique; Sd, seeds) detected by qRT-PCR. AtPARP1 and AtpARP2 are expressed in all 6 organs. However, AtpARP3 is predominantly expressed in seeds but not in other organs (C) In silicon analysis of AtPARP1, 2, and AtpARP1. The figures were obtained from Arabidopsis cFP Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb cgi) with indicated AGI numbers. Winter et al., 2007. Plant One 2(8): e1718.

(TIF)

S5 Fig. In vitro and in vivo activity of PARPs and PARGs. (A) The in vitro PARP activity of human HsPARG1 and Arabidopsis AtPARG2 detected by a β-PAR Western blot. (B) In vitro enzymatic activity of His6-tagged AtPARG1 and AtPARG2, His6-AtPARG1, but not His6-AtPARG2, hydrolyzed PAR polymers from self-modified MBP-AtPARG2 shown as the disappearance of smear detected by β-PAR antibody. (C) GST-AtPARG1 hydrolyzes PAR polymers from self-modified AtPARG2 and HsPARG1, and agg2 mutation (G450R) blocks its activity. (D) HsPARGG867R, the corresponding mutation in aggie2, abolishes its PARG activity towards self-modified AtPARG2. (E). In vivo PAR level in Col-0, atparg1, atparg2 and atparg1/2. Nuclear protein extracts were isolated, dotted onto nitrocellulose membrane, probed with β-PAR antibody (left), and quantified with ImageJ software (right). Amount of nuclear proteins was normalized to the signal of β-Histone H3 antibody WB. The atparg1 mutant accumulates higher PAR polymers than Col-0; however, PAR polymer level in atparg2 is comparable with that in Col-0.

(TIF)

S6 Fig. Comparison of AtPARG1 and AtPARG2 amino acid sequences. The alignment was generated with *Multiple sequence alignment with hierarchical clustering* F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890. (http://multalin.toulouse.inra.fr/multalin/)

(TIF)

S7 Fig. The atparg1/2 mutant is more susceptible to **P**st DC3000 infection. WT and atparg1/2 double mutant plants were hand-inoculated with **P**st DC3000 at OD_{600} = 5 × 10^{-3}, and the bacterial counting was performed 3 days post-inoculation (dpi). The data are shown as mean ± se from three independent repeats with Student's t-test. * indicates p<0.05 when compared to WT (Left panel). The disease symptom is shown at 3 dpi (right panel).

(TIF)

S8 Fig. Sequence alignment of PARG signature motif among PARGs from different species, thale cress (**Arabidopsis thaliana**), At), lyrate rockcress (**Arabidopsis lyrata**, Al), poplar (**Populus trichocarpa**, Pt), potato (**Solanum tuberosum**, S), tomato (**Solanum lycopersicum**, Sl), maize/Zea mays, Zm), sorghum (**Sorghum bicolor**, Sb), rice (**Oryza sativa**, Os), mosis(**Physcomitrella patens**, Pp), rat (**Rattus norvegicus**, Rn), mouse(**Mus musculus**, Mm), human (**Homo sapiens**, Hs), fruit fly (**Drosophila melanogaster**, Dm). The PARG signature motif is labeled in red.

(TIF)

S1 Table Cloning and point mutation primers

(S1 Table)

S2 Table qRT-PCR primers

(S2 Table)

Acknowledgments

We thank Salk Institute and ABRC for the **Arabidopsis** T-DNA insertion lines, Dr. Sylvia de Pater for atparg1/2 double mutants, Drs. Ivan Abel and Rita Westen-Goldie for His6-AtPARG1 and His6PARGs constructs (pET28a-HsPARG1 and pET28a-HsPARG2), Dr. Hidashi Koika for streptavidin-HHR, Drs. Andrew Bent and Junji Song for discussions, comments on the manuscript and sharing the data before publication.

Author Contributions

Conceived and designed the experiments: BF LS PH. Performed the experiments: BF CL MVVdO ACI KB BL. Analyzed the data: BF CL MVVdO GAdSF LS PH. Wrote the paper: BF LS PH.

References

1. Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60: 379–406.
2. Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of receptors and their associated kinase BAK1. J Biol Chem 285: 9444–9451.
3. Macho AP, Zipfel C (2014) Plant PRRs and the activation of innate immune receptors. Annu Rev Plant Biol 60: 379–406.
4. Schulze B, Mentzel T, Jehle AK, Mueller K, Beeler S, et al. (2010) Rapid development and immunity. Eur J Cell Biol 89: 169–174.
5. Lin W, Li B, Lu D, Chen S, Zhu N, et al. (2014) Tyrosine phosphorylation of Arabidopsis LRR receptor-like protein kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. Proc Natl Acad Sci U S A 107: 496–501.
6. Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, et al. (2011) The Arabidopsis leucine-rich repeat receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 23: 2440–2455.
7. Postel S, Kufner I, Beuter C, Mazzotta S, Schwedt A, et al. (2010) The Pseudomonas syringae type III effector AvrRps4 targets a Arabidopsis root cell wall receptor. J Biol Chem 285: 9444–9451.
8. Heese A, Hann DR, Gimenez-Ibanez S, Jones AM, He K, et al. (2007) Functional analysis of phosphorylation residues of the Arabidopsis BOTRYTIS-INDUCED KINASE1. Protein & Cell 4: 771–781.
9. Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, et al. (2011) The Arabidopsis leucine-rich repeat receptor kinases BAK1/BERK1 and BIK1/BERK1 are required for innate immunity to hemibiotrophic and biotrophic pathogens. Plant Cell 23: 2440–2455.
10. Lin W, Li B, Lu D, Chen S, Zhu N, et al. (2014) Tyrosine phosphorylation of protein kinase complex BAK1/BERK1 mediates Arabidopsis innate immunity. Proc Natl Acad Sci U S A 111: 3632–3637.
11. Xu JH, Wei XC, Yan LM, Liu D, Ma YY, et al. (2013) Identification and functional analysis of phosphorylation residues of the Arabidopsis BOR1-INDUCED KINASE1. Protein & Cell 4: 771–781.
Adams-Phillips L, Briggs AG, Bent AF (2010) Disruption of Poly(ADP-ribose) polymerase superfamily in plants controls leaf senescence. Plant Cell 22: 183–193.

Arvidson L, Koistin EV (2000) SAP - a putative DNA-binding motif involved in chromosomal organization. Trends Biochem Sci 25: 112–114.

Le May N, Ilis I, Ame JC, Zhovmer A, Biard D, et al. (2012) Poly(ADP-ribose) polymerase superfamily in plants. Cell Mol Life Sci 69: 175–189.

Kraus WL, Kraus WL (2012) Poly(ADP-ribose) glycohydrolase regulates retinoic acid receptor-mediated gene expression. Mol Cell 40: 785–790.

Krishnakkumar R, Gamble MJ, Frizzell KM, Berrocal JG, Kininis M, et al. (2008) Reciprocal binding of PARP-1 and histone H1 at promoters specifies transcriptional outcomes. Science 319: 819–821.

Krishnakkumar R, Kraus WL. (2010) PARP-1 regulates chromatin structure and transcription through a KDM5B-dependent pathway. Mol Cell 39: 736–746.

Wang Y, Li J, Hou S, Wang X, Li Y, et al. (2010) A Pseudomonas syringae effector, AvrRps4, induces DNA double-strand breaks whose abundance is reduced by plant defense responses. PLoS Pathog 10: e1004030.

Song, J. Bent AF (2014) Microbial pathogens trigger host DNA double-strand breaks whose abundance is reduced by plant defense responses. PLoS Pathog 10: e1004030.

Elbak S, Nevelson KJ, Steger M, Hartung ML, Hottinger MO, et al. (2011) Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells. Proc Natl Acad Sci U S A 108: 14949–14949.

Yan S, Wang W, Marques J, Mohan R, Saleh A, et al. (2013) Salicylic acid activates DNA damage responses to potenti ate plant immunity. Mol Cell 52: 602–610.

Cohen-Armon M, Visochek L, Rozenblat D, Kalal A, Geistrikh I, et al. (2007) DNA-dependent PARP-1 activation by phosphorylated E3 ubiquitin ligase Elke activity: a link to histone acetylation. Mol Cell 25: 297–308.

Kraus WL, Hottinger MO (2013) PARP-1 and gene regulation: progress and puzzles. Mol Aspects Med 34: 1109–1123.

He P, Shan L, Lin NC, Martin GB, Kemmerling B, et al. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell 125: 563–575.

Luna E, Pastor V, Robert J, Flores V, Mauch-Mani B, et al. (2011) Callose deposition: a multifaceted plant defense response. Mol Plant Microbe Interact 24: 183–193.

51. He P, Shan L, Martin GB, Kemmerling B, et al. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell 125: 563–575.

52. Toller IM, Neelsen KJ, Steger M, Hartung ML, Hottinger MO, et al. (2011) Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells. Proc Natl Acad Sci U S A 108: 14949–14949.

53. Yan S, Wang W, Marques J, Mohan R, Saleh A, et al. (2013) Salicylic acid activates DNA damage responses to potentiate plant immunity. Mol Cell 52: 602–610.

54. Cohen-Armon M, Visochek L, Rozenblat D, Kalal A, Geistrikh I, et al. (2007) DNA-dependent PARP-1 activation by phosphorylated E3 ubiquitin ligase Elke activity: a link to histone acetylation. Mol Cell 25: 297–308.

55. Kraus WL, Hottinger MO (2013) PARP-1 and gene regulation: progress and puzzles. Mol Aspects Med 34: 1109–1123.

56. He P, Shan L, Lin NC, Martin GB, Kemmerling B, et al. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell 125: 563–575.

57. Luna E, Pastor V, Robert J, Flores V, Mauch-Mani B, et al. (2011) Callose deposition: a multifaceted plant defense response. Mol Plant Microbe Interact 24: 183–193.

58. Li GJ, Nasar V, Yang XH, Li W, Liu B, et al. (2011) Arabidopsis poly(ADP-ribose) glycohydrolase 1 is required for drought, osmotic and oxidative stress responses. Plant Science 180: 283–291.

59. Asai T, Tena G, Pestikida J, Willmann MR, Chin WU, et al. (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415: 977–983.

60. He P, Shan L, Lin NC, Martin GB, Kemmerling B, et al. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell 125: 563–575.