Bacteria-derived Peptidoglycans Constitute Pathogen-associated Molecular Patterns Triggering Innate Immunity in Arabidopsis*§

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Pathogen-associated molecular pattern (PAMP)-triggered immunity constitutes the primary plant immune response that has evolved to recognize invariant structures of microbial surfaces. Here we show that Gram-positive bacteria-derived peptidoglycan (PGN) constitutes a novel PAMP of immune responses in Arabidopsis thaliana. Treatment with PGN from Staphylococcus aureus results in the activation of plant responses, such as medium alkalization, elevation of cytoplasmic calcium concentrations, nitric oxide, and camalexin production and the post-translational induction of MAPK activities. Microarray analysis performed with RNA prepared from PGN-treated Arabidopsis leaves revealed enhanced transcript levels for 236 genes, many of which are also altered upon administration of flagellin. Comparison of cellular responses after treatment with bacteria-derivative PGN and structurally related fungal chitin indicated that both PAMPs are perceived via different perception systems. PGN-mediated immune stimulation in Arabidopsis is based upon recognition of the PGN sugar backbone, while muramyl dipeptide, which is inactive in this plant, triggers immunity-associated responses in animals. PGN adds to the list of PAMPs that induce innate immune programs in both plants and animals. However, we propose that PGN perception systems arose independently in both lineages and are the result of convergent evolution.

The innate immune system is a host defense mechanism that is evolutionarily conserved from insects to humans and is mainly involved in the recognition and control of the early stage of infection in all animals (1). Over the last decade, it has become increasingly evident that also plants have acquired the ability to recognize “non self” via sensitive perception systems for components of microorganisms called pathogen-associated molecular patterns (PAMPs)2 (2–4). As classically defined, PAMPs are highly characteristic of potentially infectious microbes, but are not present in the host. In addition, such patterns are often vital for microbial survival and are therefore not subject to mutational variation. PAMPs that trigger innate immune responses in various vertebrate and non-vertebrate organisms include lipopolysaccharides (LPS) from Gram-negative bacteria, eubacterial flagellin, viral, and bacterial nucleic acids, fungal cell wall-derived glucans, chitins, mannans, or proteins and peptidoglycans (PGN) from Gram-positive bacteria (5–8).

Peptidoglycan (PGN) is an essential and unique component of the bacterial envelope that provides rigidity and structure to the bacterial cell. Virtually all bacteria contain a layer of PGN, but the amount, location, and specific composition vary. PGN is a polymer of alternating N-acetylmuramic acid (GlcNAc) and N-acetyl-muramic acid (MurNAc) residues in β-1–4 linkage which are cross-linked by short peptides (9, 10). The glycan chains display little variation among different bacterial species while the peptide subunit and the interpeptide bridge reveal species specific differences. PGN from Staphylococcus aureus belongs to the 1-lysine (Lys)-type, which is primarily found in Gram-positive bacteria whereas meso-diaminopimelate (Dap)-type PGN is typical for many Gram-negative bacteria.

As PGNs are located on most bacterial surfaces they constitute excellent targets for recognition by the innate immune system. Indeed, PGN is known for a long time to promote an innate immune response in vertebrates and insects (11–13), and a breakdown product of PGN, muramyl dipeptide (MurNAc-L-Ala-D-Glu; MDP) was found to be the minimal chemical structure required for PAMP activity in mammals (14). PGN is perceived in animals via various pattern recognition receptors (PRRs), including scavenger receptors, nucleotide-binding oligomerization domain-containing proteins (NODs), a family of peptidoglycan recognition proteins (PGRPs), PGN-lytic enzymes and Toll-like receptor TLR2 (15–19).

Remarkable similarities have been uncovered in the molecular mode of PAMP perception in animals and plants (2, 20, 21). Perception of flagellin in Arabidopsis was shown to be depend
ent on FLS2, a plasma membrane-located receptor-like kinase protein with extracellular leucine-rich repeats (LRR-RLKs) (22). The extracytoplasmic LRR-domain of FLS2 thereby resembles the structure of the extracytoplasmic domain of human TLR5, which also recognizes bacterial flagellin as a PAMP (23). Generally, transmembrane LRR proteins appear to be a common element in PAMP perception in animal and plant systems. In mammals, 11 TLRs have been identified so far and a second plant LRR-RLK, EFR, was described to recognize the bacterial elongation factor Tu (EF-Tu) (24, 25). Interestingly, the LRR-containing cytoplasmic PGN receptors NOD1 and NOD2 in mammals (26) are structurally similar to the cytoplasmic LRR-containing pathogen resistance proteins in plants that mediate plant cultivar-specific, effector-triggered immunity (ETI) (2, 4).

Here we present evidence that PGNs mediate the activation of innate defense responses in the model plant Arabidopsis thaliana in addition to their well established role as a PAMP in vertebrates and insects. Treatment with PGN from the phytopathogenic Gram-positive bacterium S. aureus (27) results in the activation of plant defense responses such as medium alkalization, elevation of cytoplasmic calcium concentrations, NO production, the activation of MAPKs, the accumulation of camalexin and the induction of various defense-related genes. Interestingly, even though a comparison of the defense responses triggered by PGN and the structurally closely related glycan chitin showed a strong overlap, our results indicate that PGN and chitin engage different perception systems. Moreover, we identify the PGN glycan backbone as the PAMP-active part in PGN. This is in contrast to mammals in which MDP was shown to be the minimal structural requirement for PAMP activity (14).

**EXPERIMENTAL PROCEDURES**

**Materials**—Flg22 peptide and hydrolyzed chitin fragments were described previously (25). MDP, muramidic acid, the pentapeptide Alad-γ-Lys-D-Ala-D-Ala, lipoteichoic acid from S. aureus, PGN from Bacillus subtilis and Streptomyces ssp. and LPS from Pseudomonas aeruginosa and Escherichia coli were obtained from Sigma and dissolved in water at a concentration of 10 mg/ml. PGN from E. coli was purchased from InvivoGen (San Diego, CA). LPS from Burkholderia cepacia was prepared as described (28). The lipopeptides PamCys, PamCy, and PamCys were a kind gift from emc microcollections (Tübingen, Germany).

**Plant Growth Conditions**—PR-1:GUS transgenic (29), pMAQ2 aerquin-transgenic (30, 31), or wild type A. thaliana Columbia-0 (Col-0) plants were grown on soil for 5–6 weeks as described (32). Dark grown cell cultures of Arabidopsis Ler were maintained as described (33) and were used for experiments 5–6 days after subculture.

**Peptidoglycan Preparation**—PGN from S. aureus SA113 (ATCC 35556) and sortase deletion mutant (SA113ΔsrtA)3 was purified as described earlier (34, 35). Briefly, cells from stationary phase cultures were harvested by centrifugation at 3,000 × g for 30 min, boiled with 5% SDS for 30 min and broken with glass beads. Insoluble polymeric PGN was harvested by centrifugation at 30,000 × g for 30 min and washed several times with lukewarm water to remove SDS. Broken cell walls were suspended in 100 mM Tris-HCl, pH 7.2, and treated with 10 μg/ml DNase and 50 μg/ml RNase A for 2 h and subsequently with 100 μg/ml trypsin for 16 h at 37 °C. To remove wall teichoic acid, the PGN preparations were incubated with 48% hydrofluoric acid (HFA) for 48 h at 4 °C. PGN was harvested by centrifugation at 30,000 × g for 30 min and washed several times with water for complete removal of HFA. Further treatment of PGN included 8 M LiCl, 10 M EDTA and acetone to remove residual protein and LPS contamination. PGN was finally washed several times with water and lyophilized. HPLC and mass spectrometry analysis of soluble PGN was carried out as described (34), with following modifications. Purified PGN (1 mg/ml) was suspended in 100 mM sodium phosphate buffer, pH 6.8, and digested with mutanolysin (50 μg/ml), lysozyme (10 μg/ml), or both for 16 h at 37 °C. Digestion was terminated by boiling the samples at 90 °C for 10 min followed by centrifugation.

Desalting of soluble muropeptides was performed by reverse phase HPLC using a Reprosil-Pur ODS-3 column (5 μm; 250 × 20 mm; Dr. Maisch). Muropeptides were eluted in a step gradient at a flow rate of 10 ml/min starting from water for 10 min to 100% methanol for the next 20 min. Muropeptides were detected at 210 nm. PGN peaks were collected and concentrated in a rotary evaporator to remove excess methanol. Finally, PGN preparations were lyophilized and stored at −20 °C.

**Histochemical GUS Detection**—For the histochemical detection of β-glucuronidase (GUS) enzyme activity whole leaves of PR-1:GUS transgenic Arabidopsis (29) were placed in 1 ml of 50 mM sodium phosphate, pH 7, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, pH 8, 0.1% Triton X-100, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc, X-Gluc-DIRECT). After vacuum infiltration, the leaves were incubated at 37 °C overnight, and chlorophyll was subsequently removed by several washings in 70% ethanol.

**Medium Alkalization, NO, and Camalexin Detection**—Medium alkalization in A. thaliana Ler cell suspensions was measured in 2-ml aliquots as described (25). Nitric oxide synthesis in cell cultures was analyzed as described (36). Camalexin production in plants was quantified by reverse phase HPLC (LiChroCART 250–4, RP-18, 5 μm, Merck; 1 ml/min; MeOH/H₂O (1:1) for 2 min, followed by a 10 min linear gradient to 100% MeOH, followed by 3 min 100% MeOH) (37). The peak at 12 min was identified as camalexin by comparison with an authentic standard with respect to retention time and UV spectrum (photodiode array detector, Dionex) and quantified using a Shimadzu F-10AXL fluorescence detector (318 nm excitation, 370 nm emission) and by UV absorption at 318 nm.

**Calcium Measurements**—Cytosolic calcium concentrations were measured by calcium-induced aerquin luminescence using transgenic Arabidopsis pMAQ2 plants expressing cytosolic apoaequorin under the control of the CaMV 35S promoter (30, 31). Mature leaves of 5-week-old plants were cut into 1-mm strips and floated on 100 μl of water supplemented with 10 μM coelenterazine (native coelenterazine, 5 μM stock in methanol, 3 G. Thumm and F. Götz, unpublished data.
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Invitro) in a 96-well plate (4 strips/well, n > 3). For aequorin reconstitution plates were incubated in the dark for at least 4 h. Luminescence was measured in a Luminoskan Ascent 2.1 luminometer (Labsystems) and recorded in integration intervals of 10 s. After 60 s recording, PAMPs were applied by addition of 50 μl of a 3-fold concentrated solution in water and measurements continued for the indicated time. Controls were performed by addition of an equal volume of water. Remaining aequorin was discharged by automatic injection of 1 volume of 2 M CaCl2/20% ethanol and luminescence was recorded for another 8–10 min until values were within 1% of the highest discharge value. Relative luminescence values were calculated and converted into actual Ca2+ concentrations as described (38).

**MAPK Activity Assay**—After elicitor treatment, leaves were immediately frozen in liquid nitrogen and MAP kinase activity was determined by in-gel kinase assays using myelin basic protein (MBP, Sigma) as substrate as described previously (39).

**RNA Isolation and Reverse Transcription-PCR**—Total RNA from leaves was isolated using the Tri Reagent method according to the manufacturer’s recommendations (Sigma). First-strand cDNA was synthesized from 1 μg of total RNA using RevertAid H minus MuLV Reverse Transcriptase (Fermentas). RT-PCR was performed as described previously (40) using gene-specific primers (supplemental Table S1). Except for EF1α, which was amplified with 25 PCR cycles, all other PCRs were performed with 30 cycles.

**Microarray Experiments**—Microarray experiments were performed on A. thaliana Col-0 plants infiltrated with 100 μg/ml PGN or water as a control. Affymetrix ATH1 high density oligonucleotide gene arrays were used for triplicate hybridizations of each biological sample. Global analysis of temporal gene expression was performed by subjecting the absolute expression values for scaling using the Affymetrix MAS5.0 software. Scaled mean values of expression were imported into Genespring software (version 7.2, Agilent Technologies, Waldbronn, Germany) using a gcRMA (41) plug-in normalization tool prior to data analysis. Means of three replicate values for each data set were analyzed for stimulus-induced differential gene expression. Data sets with expression levels below 50 were excluded from comparative analyses (noise level of expression cut-off). Genes were considered as up- or down-regulated if their mean expression levels deviated more than 2-fold from that of the non-elicited control samples.

### RESULTS

**Staphylococcal PGN Acts as a PAMP in Arabidopsis**—Despite the well established role of PGN as a PAMP in animal innate immunity, surprisingly little is known about PGN perception in plants. We thus inquired about the ability of plants to recognize and respond to PGN from the Gram-positive bacterium S. aureus, that is found as root-associated bacterium in the rhizosphere of many plants and was recently shown to be pathogenic on A. thaliana (27, 42, 43). A typical and well characterized plant response to pathogen infection or treatment with PAMPs is the induction of genes encoding pathogenesis-related (PR) proteins (44), such as PR-1. To facilitate the detection of gene induction, we used a transgenic PR-1:GUS reporter line, in which the PR-1 promoter is fused to the β-glucuronidase gene from E. coli (29, 45). As shown in Fig. 1A, treatment with heat-killed S. aureus cells or purified PGN resulted in a strong PR-1:GUS expression, similar to that observed with the elicitor-active 22 amino acid fragment from bacterial flagellin (Flg22) (46). We have consistently found PR-1 gene expression with all PGN preparations tested. Quantitative differences in PR-1 gene
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expression have only been observed upon infiltration into leaves of different PGN preparations, but not in assays using cultured cells (see below). Thus, these differences are most likely due to variations in the applicability of PGN into different biological samples. We next wanted to investigate which part of PGN is responsible for the induction of PR-1:GUS. Purified PGN was digested with either lysozyme, an enzyme that specifically cleaves pentaglycin interpeptide bridges in staphylococcal PGN, or with mutanolysin, which hydrolyzes glycosidic linkages between disaccharide units of PGN (Fig. 1B). After HPLC purification, soluble muropeptides were infiltrated into Arabidopsis leaves, and GUS activity was detected after 24 h. Whereas lysozyme-digested PGN was able to trigger PR-1:GUS expression, no induction was observed with mutanolysin- or mutanolysin/lysozymin-digested PGN (Fig. 1C). Furthermore, treatment with smaller synthetic PGN components such as MDP, muramic acid or the pentapeptide Ala-D-Ala-D-Ala-D-Lys-Glu-Lys-D-Ala-D-Ala did not result in any PR-1:GUS induction. Taken together, these results indicate that not the protein part of PGN is perceived by Arabidopsis. Rather, sugar chains longer than the disaccharide are recognized, which is in contrast to PGN perception in vertebrates in which already MDP displays strong immunogenic activity (14).

PGN Triggers Early Plant Immune Responses—Medium alkalinization, occurring as a consequence of altered ion fluxes across the plasma membrane, is one of the earliest marker responses observed in elicitor-treated plant cells (47). In tobacco cells, addition of lyophilized Micrococcus lysodeikticus cells as well as M. lysodeikticus PGN induced a strong and rapid increase in extracellular pH (48). Here, we compared PGN-induced changes in extracellular pH in Arabidopsis cell suspension cultures with that induced by Flg22 (Fig. 2A). Flg22 treatment resulted in a rapid but transient increase in extracellular pH, reaching a maximum at about 30–40 min. In comparison, PGN induced a somewhat slower but more persistent increase in extracellular pH starting after a lag phase of about 15 min. Similar responses were obtained with commercially available PGN from the Gram-positive bacteria B. subtilis and Streptomyces spp. (supplemental Fig. S1) and the Gram-negative bacterium E. coli (supplemental Fig. S2). Medium alkalinization was dose-dependent with saturating concentrations of 100–200 μg/ml PGN (Fig. 2B). As a concentration of 100 μg/ml PGN triggered nearly maximal responses in medium alkalinization assays (see also Fig. 5D) all further experiments were conducted with this PGN concentration.

Post-translational activation of mitogen-activated protein kinase (MAPK) activity is commonly associated with plant immunity (49). PGN or Flg22 were infiltrated into Arabidopsis leaves, and MAPK activity was subsequently analyzed in an in-gel kinase assay using MBP as artificial substrate. As shown in Fig. 2C, hypoosmotic stress because of water infiltration in the control samples caused a rapid, but transient activation of two MAPK species of 44 and 46 kDa, respectively. However, PGN treatment resulted in a much stronger response, which closely resembled that obtained with Flg22, most likely representing activation of MPK3 and MPK6 (50). Another hallmark of immune responses in animals and plants is the production of nitric oxide (NO) (36). Treatment of Arabidopsis cells with Flg22 or PGN resulted in a significant increase in NO production within 30 min (Fig. 2D). Calcium is a key second messenger in signal transduction pathways to external stimuli in various organisms (51, 52). We used Arabidopsis plants expressing cytoplasmic aequorin (31) to monitor changes of cytoplasmic
Ca²⁺ levels in vivo after PAMP treatment. Luminometry of Arabidopsis leaf strips treated with Flg22 revealed a strong and rapid increase in [Ca²⁺]_{cyt} starting after a 30–40 s lag phase and peaking after 2–3 min, which was followed by a plateau phase of elevated [Ca²⁺]_{cyt} (Fig. 2E). Likewise, PGN treatment also resulted in a significant elevation of [Ca²⁺]_{cyt}; however, as observed for medium alkalinization, increase of [Ca²⁺]_{cyt} occurred more slowly, reaching almost the same [Ca²⁺]_{cyt} as the flg22-induced plateau phase at about 10 min before a gradual decrease to basal levels (Fig. 2E).

As undigested PGN is a very complex molecule, we wanted to rule out that cell wall components tightly associated with PGN act as PAMPs in addition to or instead of PGN. For example, lipopeptides and lipoteichoic acid are ligands for TLR-mediated immunity in animals (6). Lipoteichoic acid, LPS and highly abundant proteins such as flagellin or EF-Tu have also been shown to trigger immune responses in Arabidopsis (25, 36, 53). We therefore tested the synthetic lipopeptide analogs Pam₃Cys, Pam₂Cys, and PamCys (54), LPS preparations from P. aeruginosa and B. cepacea and lipoteichoic acid from S. aureus in medium alkalinization assays. All tested compounds induced a weak pH shift in Arabidopsis cell cultures but with completely different kinetics to that observed with PGN (Fig. 3B–D). Moreover, the lipopeptides Pam₃Cys, Pam₂Cys, and PamCys did not trigger PR-1:GUS reporter gene expression (Fig. 3A). Furthermore, no protein or LPS contamination was detected in PGN preparations using SDS-PAGE followed by silver staining, and proteinase K-digested PGN still induced PR-1:GUS expression (data not shown). In addition, PGN preparations from the S. aureus sortase mutant ΔsrtA still induced PR-1 expression (data not shown). These mutant bacteria are defective in covalently tethering surface proteins to PGN (55), indicating that PR-1:GUS induction was not due to contaminations of PGN preparations with PGN-associated proteins. To rule out that the PGN-induced pH shift was merely a consequence of mechanical stimulation due to the insolubility of
PGN, we also treated cells with a suspension of ground glass pasteur pipettes. This treatment, however, did not induce any medium alkalization (data not shown). Furthermore, we measured medium alkalization after treatment of cell cultures with HPLC-purified lysostaphin-, mutanolysin- or mutanolysin/lysostaphin-digested PGN. Similar to the PR-1-GUS assay (Fig. 1C), only lysostaphin-digested PGN displayed strong PAMP activity while only a marginal pH shift was induced with mutanolysin- or mutanolysin/lysostaphin-digested PGN (Fig. 3E). However, as the response to lysostaphin-digested PGN was somewhat weaker than to undigested PGN, it seems that the sugar backbone accounts for the majority, but not necessarily all, of the PAMP-activity. Altogether, these data indicate that PGN and not putative proteinaceous or other contaminants such as lipopeptides are responsible for the induction of the observed cellular responses.

Inducible PR-1-GUS expression (Fig. 1B) suggested that PGN treatment may have an impact on plant gene expression patterns. To get a comprehensive overview on PGN-induced changes in the *Arabidopsis* transcriptome we performed microarray analyses using plant material harvested 4 h after either PGN or water treatment. For comparative analysis, microarray data for the 4-h time point after Flg22 treatment were obtained from AtGenExpress experiments (56). For each treatment versus control condition, genes with an altered expression were assigned based on a one-way analysis of variance test combined with a Benjamini and Hochberg false discovery rate algorithm (cutoff of 0.05). Of the ~23,750 expressed genes represented on the Affymetrix ATH1 full genome array (57), expression of 236 genes (1%) was found to be induced more than 2-fold after PGN treatment (supplemental Table S2). Intriguingly, we observed a strong overlap of genes with altered expression when comparing PGN and Flg22 treatment (Table 1 and Fig. 4). Gene induction for randomly chosen genes was confirmed by RT-PCR analysis and all tested PGN-induced genes showed a similar expression profile after Flg22 treatment (Fig. 4).

A detailed analysis of PGN-induced genes (Table 1 and supplemental Table S2) revealed a considerable number of up-regulated genes that can be classified as being involved in signal perception, such as receptor-like kinases (18 genes) or disease resistance-like proteins (5 genes), and signal transduction, such as protein kinases (17 genes) and phosphatases (1 gene). Moreover, genes coding for typical defense-related proteins were responsive to both PGN and Flg22 treatment including chitinases (5 genes), protease inhibitors (5 genes), peroxidases (1 gene), phenylalanine ammonia lyase 1 (PAL1), and a HIN1-family protein. We also found numerous up-regulated genes with a putative function in protein degradation (U-box or F-box-domain-containing proteins, 5 genes) and transcriptional regulation (WRKY transcription factors, AP2 domain-containing transcription factors, 16 genes).

**PGN Induces Phytoalexin Production without Causing Cell Death**—While medium alkalization, NO production, and MAPK activation are early responses observed after various PAMP treatments, the production of the antimicrobial phytoalexin camalexin (58) occurs at later stages and was shown to be induced after infection with *P. syringae* (59) or upon treatment with the necrotizing *phytophthora parasitica* toxin NLP$_{pp}$ (56) and heat-killed yeast cells (60). No significant increase in camalexin levels could be detected in *Arabidopsis* leaves after Flg22 treatment. However, both PGN and NLP$_{pp}$ triggered a strong production of this phytoalexin, reaching up to 155 µg/g dry weight after 4 days of PGN treatment (Fig. 5B). In contrast to NLP$_{pp}$, which triggers cell death (32, 56), infiltration of PGN and Flg22 did not result in any macroscopic tissue damage for up to 5 days (Fig. 5A and data not shown).

**PGN and Chitin Do Not Engage the Same Perception System**—Elicitor activity of PGN depends on an intact glycan backbone (Figs. 1C and 3E), which consists of alternating N-acetylglucosamine and N-acetylmuramic acid residues. This carbohydrate backbone resembles the unbranched β1–4-linked N-acetyl-glucosamine chains of chitin. Chitin is a major component of fungal cell walls and has been shown to act as PAMP in many plant species. In *Arabidopsis*, chitin was shown to induce typical PAMP responses such as the activation of MAPK cascades (61) and alterations in protein phosphorylation (62) or gene transcription (63, 64). We compared both elicitors with respect to PR-1 expression by using both the PR-1-GUS reporter line and RT-PCR analysis, but we could not observe any chitin-induced PR-1 expression in concentrations up to 100 µg/ml (Fig. 6, A and B). Interestingly, chitin strongly induced a transient expression of At2g39530 and At1g51850, whereas inducible gene expression after PGN and Flg22 treatment was prolonged and still detectable after 24 h. Furthermore, we measured medium alkalization after PGN or chitin treatment. As shown in Fig. 6C, chitin induced a very rapid and transient pH shift similar to that observed after Flg22 addition (Fig. 2A), but clearly distinguishable from the delayed and prolonged response triggered by PGN. The difference in PGN and chitin induced defense responses suggested that both elicitors were recognized by different perception systems. To corroborate this finding, we investigated whether chitin and PGN are perceived by different perception systems using the alkalization assay. *Arabidopsis* cell cultures treated for 70 min with 100 µg/ml PGN did not show a significant further increase in extracellular pH when treated with a second dose of 1 mg/ml PGN, indicating saturation of these cells for PGN. However, these cells still responded to subsequent treatment with 100 µg/ml chitin, strongly suggesting that PGN and chitin are perceived via different receptors.

**DISCUSSION**

PGN from both Gram-positive and Gram-negative bacteria is highly immunogenic in mammals and *Drosophila* (17). Although Gram-positive bacteria have so far not been regarded as important plant pathogens, members of the species *Streptomyces* are known for a long time to cause economically important diseases such as potato scab (65). *Streptomyces* spp. are not host specific and can also infect *Arabidopsis* (66). Similarly, Gram-positive *S. aureus* causes typical bacterial disease symptoms in *in vitro* and soil-grown *Arabidopsis* plants such as water-soaked lesions and chlorosis eventually leading to plant death both upon leaf and root inoculation (27). Here we show that *Arabidopsis* is able to respond to PGN as a PAMP from both Gram-positive and Gram-negative bacteria. Typical plant immunity-associated responses were triggered such as medium alkalization, increase in [Ca$^{2+}$]$_{cyt}$, NO production, camalexin
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**TABLE 1**

| AGI number  | Gene description                                | Name          | PGN Fold change | Flg22 Fold change | p value   | p value |
|-------------|-------------------------------------------------|---------------|----------------|-------------------|-----------|---------|
| AT1G07390   | Leucine-rich repeat family protein               |               | 2.4            |                   | 0.017     | 0.039   |
| AT1G09970   | Leucine-rich repeat transmembrane protein kinase |               | 2.6            |                   | 0.009     | 3.7     |
| AT1G17590   | Leucine-rich repeat protein kinase               |               | 3.3            |                   | 0.009     | 0.011   |
| AT1G18000   | Leucine-rich repeat protein kinase               |               | 6.8            |                   | 0.007     | 60.3    |
| AT1G18200   | Leucine-rich repeat protein kinase               |               | 8.8            |                   | 0.011     | 83.9    |
| AT1G18500   | Leucine-rich repeat protein kinase               |               | 15.5           |                   | 0.007     | 260     |
| AT1G18900   | Leucine-rich repeat protein kinase               |               | 5.6            |                   | 0.011     | 64.2    |
| AT1G53400   | Leucine-rich repeat protein kinase               |               | 2.1            |                   | 0.017     | 3.5     |
| AT2G02220   | Leucine-rich repeat protein kinase               |               | 2.3            |                   | 0.011     | 13.6    |
| AT2G19190   | Light/senescence-responsive LRR protein kinase   | FRK1          | 4.9            |                   | 0.011     | 196.1   |
| AT4G08850   | Leucine-rich repeat transmembrane protein kinase |               | 2.5            |                   | 0.017     | 4.3     |
| AT2G37710   | Lectin protein kinase                            |               | 2.2            |                   | 0.017     | 5.8     |
| AT5G35370   | Lectin protein kinase                            |               | 2.1            |                   | 0.027     | 4.5     |
| AT1G61360   | S-locus lectin protein kinase                    |               | 2.1            |                   | 0.017     | 2.8     |
| AT4G21390   | Seven transmembrane MLO family protein kinase    | MLO12         | 8.8            |                   | 0.011     | 38.7    |
| AT2G39200   | Seven transmembrane MLO family protein kinase    | MLO12         | 19             |                   | 0.007     | 77.4    |
| AT3G45290   | Seven transmembrane MLO family protein kinase    | MLO3          | 2.9            |                   | 0.035     | 23.5    |
| AT2G39660   | Protein kinase family                            | BIK1          | 2.8            |                   | 0.031     | 3.2     |
| AT3G46280   | Protein kinase related                           |               | 15.9           |                   | 0.007     | 264.4   |
| AT4G11330   | Mitogen-activated protein kinase (MPK5)          | MPK5          | 2.4            |                   | 0.045     | 2.5     |
| AT4G22190   | Protein kinase family                            | MPK17         | 2.4            |                   | 0.015     | 3.5     |
| AT4G23210   | Protein kinase family                            | APK1b         | 2.2            |                   | 0.017     | 3.2     |
| AT4G23220   | Protein kinase family                            |               | 2.1            |                   | 0.007     | 4.7     |
| AT4G23300   | Protein kinase family                            |               | 2.9            |                   | 0.010     | 5.6     |
| AT5G20050   | Protein kinase family                            |               | 3.1            |                   | 0.013     | 10.2    |
| AT5G44430   | Calcium-dependent protein kinase, CDPK           |               | 2.5            |                   | 0.039     | 6.5     |
| AT5G90920   | Protein kinase family                            |               | 2.1            |                   | 0.019     | 3.9     |
| AT5G61560   | Protein kinase family                            |               | 3              |                   | 0.017     | 22.1    |
| AT2G40180   | Protein phosphatase 2C                           | PP2C5         | 6.3            |                   | 0.019     | 9       |
| AT3G45420   | Seven transmembrane MLO family protein 3 (MLO3)  | MLO3          | 2.9            |                   | 0.035     | 7.4     |
| AT2G39200   | Seven transmembrane MLO family protein 12 (MLO12)| MLO12         | 19             |                   | 0.007     | 77.4    |
| AT1G65390   | Disease resistance protein (TIR class)           | ATP2-A5       | 8.8            |                   | 0.013     | 51.97   |
| AT4G09420   | Disease resistance protein (TIR-NBS class)       |               | 3.5            |                   | 0.018     | 6.5     |
| AT5G44910   | TIR domain-containing protein                    |               | 6.5            |                   | 0.011     | 38.7    |
| AT1G02360   | Chitinase                                        |               | 2.9            |                   | 0.011     | 18.8    |
| AT1G43620   | Chitinase                                        |               | 4.2            |                   | 0.007     | 5.1     |
| AT1G43590   | Chitinase                                        |               | 5.1            |                   | 0.033     | 2.9     |
| AT1G43820   | Class IV chitinase (CHIV)                        | AtEP3         | 3.7            |                   | 0.028     | 23.5    |
| AT1G01700   | Chitinase                                        |               | 4.3            |                   | 0.010     | 24.8    |
| AT2G35980   | Harpin-induced protein (YLS9) / HIN1 family protein | HIN1         | 2.7            |                   | 0.043     | 6.8     |
| AT2G37040   | Phenylalanine ammonia-lyase 1 (PAL1)            | PAL1          | 3              |                   | 0.049     | 6.5     |
| AT2G20400   | Immediate-early fungal elicitor family protein   |               | 3.2            |                   | 0.014     | 9.7     |
| AT5G64120   | Peroxidase                                       |               | 7.5            |                   | 0.010     | 25.4    |
| AT2G38870   | Protease inhibitor                               |               | 3.2            |                   | 0.011     | 6.3     |
| AT4G12470   | Protease inhibitor/seed storage/lipid transfer protein (LTP) | PEARLI   | 3.9            |                   | 0.011     | 9.9     |
| AT4G12500   | Protease inhibitor/seed storage/lipid transfer protein (LTP) | PEARLI   | 46.1           |                   | 0.028     | 144.3   |
| AT4G22470   | Protease inhibitor/seed storage/lipid transfer protein (LTP) | PEARLI   | 11.5           |                   | 0.011     | 18.9    |
| AT1G66160   | U-box domain-containing protein                  |               | 4.4            |                   | 0.007     | 8.7     |
| AT2G35930   | U-box domain-containing protein                  |               | 7.5            |                   | 0.011     | 63.1    |
| AT1G52450   | U-box domain-containing protein                  |               | 21.2           |                   | 0.010     | 154.8   |
| AT1G15670   | Kelch repeat-containing F-box family protein    |               | 3.8            |                   | 0.035     | 19.1    |
| AT5G43190   | F-box family protein (FBX6)                      | FBX6          | 2.2            |                   | 0.033     | 3.6     |
| AT1G62300   | WRKY family transcription factor                | WRKY6         | 2.5            |                   | 0.011     | 5.7     |
| AT1G80840   | WRKY family transcription factor                | WRKY40        | 2.2            |                   | 0.018     | 8.2     |
| AT2G23320   | WRKY family transcription factor                | WRKY15        | 2.3            |                   | 0.019     | 5.2     |

*Data derived from one-way analysis of variance combined with a Benjamini and Hochberg false discovery rate calculation are given. p values greater than the threshold of 0.05 are indicated by >0.05.*
accumulation, MAPK activation and extensive reprogramming of the transcriptome (Figs. 2–5). Immune responses were triggered with PGN concentrations of about 100 μg/ml, which is in accordance to the amounts that are necessary to stimulate immune responses in mammals and Drosophila (16, 67).

Although responses to Flg22 were often stronger than those to PGN, the effects of both elicitors were quantitatively similar. In particular, early cellular responses such as medium alkalinization, NO production and MAPK activation were basically indistinguishable upon stimulation with PGN or Flg22 (Fig. 2). More evidence that responses to PGN and Flg22 are comparable is the large overlap of alterations in gene expression observed by microarray analysis (Fig. 4 and Table 1). Importantly, genes encoding proteins implicated in pathogen recognition, such as receptor-like kinases and resistance proteins, resistance signaling like WRKY transcription factors and plant defense execution like PR-proteins were found to be co-induced, suggesting that both signals are perceived as equivalent determinants of microbial non-self by the plant and similarly trigger activation of the plant surveillance system. Altogether, PGN, as a constitutive surface component of many bacterial cells, must be added to the list of PAMPs with immunogenic activity in both plants and animals.

PGN, flagellin, and chitin act as PAMPs inducing largely overlapping response patterns. However, defense responses were not identical as has been proposed for the two proteinaceous elicitors flagellin and EF-Tu (24). PGN treatment resulted for instance in camalexin production whereas application of Flg22 did not (Fig. 5B). Additionally, the pH shift induced by chitin closely resembled that induced by Flg22 (compare Figs. 2A and 6C), but chitin treatment did not result in any PR-1 transcription (Fig. 6, A and B). Comparison of the microarray data indicated a strong overlap of PGN- and Flg22-responsive genes; however, Flg22 induced approximately three times as many genes as PGN ((56) and supplemental Table S2). Moreover, only 133 of a total of 1168 chitin-induced genes (63)

### TABLE 1—continued

| AGI number   | Gene description                  | Name | PGN Fold change | p value | Flg22 Fold change | p value |
|--------------|-----------------------------------|------|----------------|---------|-----------------|---------|
| AT2G24570    | WRKY family transcription factor  | WRKY 17 | 2.5  | 0.011 | 20.6 | 0.015 |
| AT2G38470    | WRKY family transcription factor  | WRKY 33 | 2.8  | 0.023 | 7   | >0.05 |
| AT4G01720    | WRKY family transcription factor  | WRKY 47 | 4.1  | 0.016 | 10.7 | 0.007 |
| AT4G18170    | WRKY family transcription factor  | WRKY 28 | 3.6  | 0.009 | 7.7  | 0.020 |
| AT4G23810    | WRKY family transcription factor  | WRKY 33 | 3.2  | 0.011 | 5.9  | >0.05 |
| AT4G24240    | WRKY family transcription factor  | WRKY 7  | 3.9  | 0.019 | 13.8 | 0.029 |
| AT4G31550    | WRKY family transcription factor  | WRKY 11 | 2.8  | 0.019 | 6.8  | 0.004 |
| AT5G49520    | WRKY family transcription factor  | WRKY 48 | 5.3  | 0.019 | 12.1 | 0.012 |
| AT3G23250    | Myb family transcription factor   | MYB15  | 5.1  | 0.028 | 26.7 | 0.008 |
| AT1G63820    | AP2 domain-containing transcription factor, ERF-family | RAV2 | 2.6  | 0.029 | 3.7  | 0.013 |
| AT1G68440    | AP2 domain-containing protein     | CEJ1   | 2.1  | 0.025 | 5.4  | 0.036 |
| AT1G63820    | AP2 domain-containing transcription factor, ERF-family | ERF9 | 3.8  | 0.038 | 6.2  | 0.005 |
| AT1G68440    | Integral membrane protein         | Others | 3.5  | 0.019 | 288.1 | >0.05 |
| AT4G15610    | Integral membrane protein         |       | 2.7  | 0.019 | 8.6  | 0.009 |

*Genes with a significant induction after 1 h of Flg22 treatment (56).*

![FIGURE 4. **PGN activates expression of various genes.** Arabidopsis leaves were infiltrated with 1 μM Flg22, 100 μg/ml PGN, or water as a control. Leaves were harvested at the indicated time points and total RNA was isolated and subjected to RT-PCR with specific primers for PGN-induced genes. Equal cDNA amounts were controlled by amplification of the constitutively expressed EF1a gene.](image)

![FIGURE 5. **PGN induces camalexin production, but no cell death.** A, Arabidopsis leaves were infiltrated with 1 μM Flg22, 100 μg/ml PGN, 2 μM NLP<sub>PP</sub>, or water as a control and pictures were taken 2 days after infiltration. B, camalexin accumulation in six independent leaves was determined at 2 days (gray bars) or 4 days (black bars).](image)
differences in PAMP perception have also been described in animals. Differential responses to various TLR ligands was partially attributed to a selective usage of certain adaptor molecules linked to TLRs followed by differential activation of transcription factors (8, 68). It can be assumed that each PAMP triggers specific sets of both individual as well as generic cellular responses with putative cross-talk of the signaling pathways. Another explanation for the observed differences in PAMP-induced downstream responses could be that some late defense-related responses such as PR-1 induction or camalexin production require a certain threshold of preceding events. Hence, strength, kinetics, and duration of the induction would be important for triggering those late cellular responses. We could for instance observe that PGN triggered a sustained pH shift, whereas the response to Flg22 was stronger and faster, but more transient (Fig. 2A) and thus possibly not lasting long enough to induce camalexin production (Fig. 5). Moreover, different PAMP perception systems might rely on different classes of receptor molecules, each possibly initiating a specific subset of downstream responses. Intriguingly, proteinaceous PAMPs such as flagellin and EF-Tu not only induce nearly identical plant responses but are also perceived by the same kind of receptors, the two LRR-RLKs FLS2 and EFR (22, 24). The identity of the corresponding PGN receptor(s) and its nature are outstanding questions and will be the focus of future research.

In animal systems, it has been shown that compounds that are associated with PGN, such as lipoteichoic acid or lipopeptides, also possess immunostimulatory activity (6). For S. aureus it has been shown that lipoproteins play a major role in promoting immune responses in various human cell lines, whereas lipoteichoic acid has, if at all, only a minor role (69, 70). However, we could rule out such PGN contaminations by performing a number of experimental controls: (i) Medium alkalization assays in Arabidopsis cell cultures indicated that although lipoteichoic acid, lipopolysaccharides, or lipopeptides induced weak pH shifts, this response showed differences in kinetics compared with PGN (Fig. 3, B–D). (ii) PR-1:GUS expression assays revealed LPS and lipopeptides as rather weak inducers of defense gene expression in Arabidopsis (Fig. 3A). (iii) Lysostaphin-digested, HPLC-purified PGN retained PAMP-activity (Fig. 3E). (iv) PGN-associated PAMP activity was not lost after heating or protease-digestion, and (v) PGN preparations of S. aureus sortase mutants, which lack PGN-associated proteins, were still able to induce PR-1 gene expression (data not shown). Altogether, these data indicate that the observed responses in Arabidopsis must be attributed to the recognition of PGN rather than to factors associated with PGN.

Interestingly, cellular responses in Arabidopsis were only induced by whole PGN preparations or PGN that was digested with lysostaphin which creates long PGN glycan chains that are no longer interconnected by peptide stems. In contrast, smaller PGN constituents such as purified fragments after mutanolysin or mutanolysin/lysostaphin digestion, MDP, muramic acid or the cross-linking pentapeptide remained inactive (Figs. 1 and 3E). Our data suggest that Arabidopsis has evolved a perception system for the glycan part of PGN, which is highly conserved among all bacteria, rather than for the peptide crosslink, in which the amino acid composition can vary between bacterial
species. This is in contrast to mammals in which MDP as a natural partial structure of PGN was reported to be the minimal structure with immunostimulatory activity (14). However, we could not observe any PAMP activity for MDP in Arabidopsis when used to induce medium alkalinization or PR-1::GUS expression (Fig. 1C and data not shown). Apparently, plants and animals have different structural requirements for PGN recognition. Interestingly, Drosophila is also not responsive to MDP (12) although PGN triggers strong immune responses in this insect (71). Rather, the minimal structure required to stimulate Drosophila innate immunity is a muropeptide dimer, and monomers (GlcNAc-MurNAc-dipeptide) were completely inactive and even had an inhibitory effect (67). Moreover, tracheal cytotoxin (TCT), a tetrapeptide containing PGN fragment, was a strong inducer of mouse NOD1 whereas human NOD1 required a tripeptide for efficient sensing of PGN (72), again indicating that PGN detection systems are host-specific. Apparently, even though PGN is recognized in plants, insects, and mammals, the different lineages have evolved perception systems for distinct regions of this complex PAMP. This has also been demonstrated for flagellin, the protein subunit that builds up bacterial flagella: whereas the Arabidopsis flagellin receptor FLS2 recognizes the very conserved peptide Flg22, which is part of the so called Spike region of bacterial flagellin, a more central hypervariable peptide in the D1 region acts as PAMP in mammals (73, 74). Moreover, in mammals lipid A as the invariable part of LPS is the most potent stimulator of innate immunity (75, 76), whereas Arabidopsis perceives LPS via both the lipid A part as well as synthetic oligo-aminans, which are commonly found in the highly variable O-antigen of LPS (36, 77).

We suggest a model in which PGN was chosen as non-self determinant both in plants and animals because of its characteristics as a typical PAMP: it is widely found in bacteria, structurally stable, displayed on the cell surface and not found in eukaryotic cells. However, our results indicate that plants respond differently to PGN than animals. Therefore, despite obvious conceptual similarities in plant and animal innate immunity, PAMP perception systems in both kingdoms are most likely the result of independent, convergent evolution (3, 7, 20).

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