Amyloidogenesis of Type III-dependent Harpins from Plant Pathogenic Bacteria

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Harpins are heat-stable, glycine-rich type III-secreted proteins produced by plant pathogenic bacteria, which cause a hypersensitive response (HR) when infiltrated into the intercellular space of tobacco leaves; however, the biochemical mechanisms by which harpins cause plant cell death remain unclear. In this study, we determined the biochemical characteristics of HpaG, the first harpin identified from a Xanthomonas species, under plant apoplast-like conditions using electron microscopy and circular dichroism spectroscopy. We found that His6-HpaG formed biologically active spherical oligomers, protofibrils, and β-sheet-rich fibrils, whereas the null HR mutant His6-HpaG(L50P) did not. Biochemical analysis and HR assay of various forms of HpaG demonstrated that the transition from an α-helix to β-sheet-rich fibrils is important for the biological activity of protein. The fibrillar form of His6–HpaG is an amyloid protein based on positive staining with Congo red to produce green birefringence under polarized light, increased protease resistance, and β-sheet fibril structure. Other harpins, such as HrpN from Erwinia amylovora and HrpZ from Pseudomonas syringae pv. syringae, also formed curvilinear protofibrils or fibrils under plant apoplast-like conditions, suggesting that amyloidogenesis is a common feature of harpins. Missense and deletion mutagenesis of HpaG indicated that the rate of HpaG fibril formation is modulated by a motif present in the C terminus. The plant cytotoxicity of HpaG is unique among the amyloid-forming proteins that occur in several microorganisms. Structural and morphological analogies between HpaG and disease-related amyloidogenic proteins, such as Aβ protein, suggest possible common biochemical characteristics in the induction of plant and animal cell death.

In many Gram-negative plant pathogenic bacteria, the hrp (hypersensitive response and pathogenicity) genes, which mostly encode proteins necessary for type III protein secretion systems, are involved in the secretion of harpins (1, 2). Harpins are heat-stable, glycine-rich type III-secreted proteins that cause a hypersensitive response (HR) when filtrated into the intercellular space of tobacco leaves (1, 3, 4). The HR in plants is an early defense response that restricts the growth of plant pathogens by causing cell death. The plant HR is similar to programmed cell death, or apoptosis, in animal cells, and the biochemical changes that occur during the HR in plant cells have been well documented (5–8). Two categories of HR exist in plants, pathogen-driven and harpin protein-dependent. A typical pathogen-driven HR occurs when a pathogen carrying an avirulence gene enters the intracellular environment of a plant carrying its cognate resistance gene. The second type of HR results from the activity of bacterial harpins outside of plant cells, including those found in Pseudomonas syringae pv. syringae, Erwinia amylovora, Erwinia chrysanthemi, and Xanthomonas axonopodis pv. glycines (1, 3, 9–12). In Ralstonia solanacearum, a harpin-like protein, PopA, has been shown to induce an HR in nonhost tobacco plants (13).

Among the harpins, HrpN (3), HrpZ (9), and HrpW (11) are well characterized HR elicitors. We previously reported the identification of a harpin, HpaG, which is produced by X. axonopodis pv. glycines 8ra (1), and we discovered that an hpaG mutant lacking the ability to induce an HR was much less virulent in susceptible soybean cultivars. HpaG induces an HR in various nonhost plants, but it exhibits specificity for those plants; HpaG induces an HR in tobacco and pepper plants but not in tomato or Chinese cabbage (1). In a previous study (4), we reported that HpaG has two predicted α-helices (motifs 1 and 3) in the N- and C-terminal regions, and that a Gln and Gly repeated sequence (motif 2; QQGQGQQG) is located between the two α-helices (Fig. 1, A and B). The motif 2 region is homologous to the prion-forming domain (PrD) of a yeast prion protein, Rnq1p (14). Using mutagenesis and deletion analysis, we deduced that 23 amino acids in motif 1 are essential for inducing the HR. This was confirmed by showing that a synthetic peptide comprising these 23 amino acids was capable of eliciting an HR, and a single amino acid change at residue 50 from leucine to proline abolished this activity (4).

The mechanisms of HR induction by HpaG and other harpins are far from being completely understood. Harpins are believed to act on plant cell walls because a physical interaction...
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A

1. WNSLNTQLGANSSSFQVDPGNTSSPONGNLG1 SEKQLDQLTLTQL MAL
   1
   51  LOQSNMAEGQGGOGQGDGGGGMNPDAQGNSPSQTYGALMNI TVGDL
   2
   101  LOAQNGGFDGGFDDGGFGILVTLASDTGSMQ

B

Motif 1 missense mutant

HpaG(L50P) 1 2 3

C-terminal region deletion mutants

ΔC66 1 2 +

ΔC75 1 58 +

Motif 2 missense mutants

HpaG-C1 1 2 * +

HpaG-C2 1 2 * +

Motif 3 missense mutants

HpaG(L93P) 1 2 * +

HpaG(L101P) 1 2 * +

HpaG(C90P) 1 2 * +

HpaG(C90P-L101P) 1 2 * +

FIGURE 1. Amino acid sequence of HpaG and schematic representations of HpaG and HpaG mutants based on protein secondary structure predictions. A, underlined regions mark motifs within HpaG. Motif 1 is a predicted α-helix in the N-terminal region; motif 2 (QGQGQGQG) matches the sequence of the prion-forming domain (PrD) in the yeast prion Rnp1p (14), and motif 3 is a predicted α-helix in the C-terminal region. An asterisk marks the residue mutated in HpaG(L50P). B, representations of HpaG, HpaG peptide, and missense/mutation mutants of HpaG. The shaded regions represent motifs; the numerical labels indicate the order of the motifs within HpaG. The asterisk marks the location of the single amino acid substitution, and the closed circle in the flexible region of the missense mutants marks the location of the triple amino acid substitution; HpaG-C1 is HpaG(K61L/G62L/G63L) and HpaG-C2 is HpaG(K66L/G66L/G67L). All mutants showed the same level of activity as wild-type HpaG, except for the null HR mutant, HpaG(L50P), and HpaG(L50P) peptide.

of HrpZ with plant cell walls has been observed in P. syringae pv. syringae (15). In addition, it has been demonstrated that HrpZpsph from P. syringae pv. phaseolicola associates with synthetic bilayer membranes and mediates the formation of an ion-conducting pore that is permeable to cations (16). In addition, HrpN from E. amylovora and Pantoaea stewartii subsp. stewartii was found to depolarize plant cell membranes (17, 18), and PopA from R. solanacearum has pore-forming activity and integrates into the liposomes and membranes of Xenopus laevis (19). This unusual membrane permeability is speculated to be the cause of cellular dysfunction and death; however, additional biochemical details about harpins are required to explain how they destabilize membranes to cause HRs.

Under physiological conditions, various innocuous and soluble proteins polymerize to form insoluble amyloid fibrils. As amyloid fibrils or fibril precursors, these proteins not only lack their biological activities but may also be harmful to organisms, causing a variety of human neurodegenerative and amyloid-related diseases, such as Alzheimer disease (20, 21). Although the etiology and pathogenesis of amyloid-related diseases are not fully understood, the drastic structural changes in amyloid proteins that create their unique β-sheet fibrils are the most important events in amyloid disease (22, 23). Nonpathogenic amyloid-forming proteins have been reported in several microorganisms, including a yeast prion protein from Saccharomyces cerevisiae (14, 24, 25), chaplins from Streptomyces coelicolor (26), curli expressed by Escherichia and Salmonella spp. (27), and microcin E492 from Klebsiella pneumoniae (28).

Here, we present a Xanthomonas harpin, HpaG, as an amyloid-forming protein. Based on biochemical analyses of HpaG, we demonstrate that α-helices and amyloidogenesis are key features related to the biological activity of harpins. We also show that HpaG shares common biochemical characteristics with amyloid disease-related proteins responsible for neuronal degeneration and cell death.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The construction of the plasmids pJ14 (hpaG in pET14b), pHGM76 (hpaG[L50P] in pET14b), and pTHG67 (hpaGA66C in pET14b) have been described in previous reports (1, 4). The bacterial strains and plasmids used in this study are listed in supplemental Table S1.

Purification of HpaG and Other Harpins—We purified His6-tagged HpaG (His6-HpaG), HpaG derivatives, and other harpins (His6-HrpN, His6-HrpZ, His6-XopA, and His6-XopA(F48L/M52L)) as described previously (1). We used Superdex-75 HR 10/30 or Superdex-200 HR 10/30 (Amersham Biosciences) to obtain highly purified His6-HpaG. The concentration of the purified protein was determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard. The HpaG and HpaG(L50P) peptides were synthesized as described previously (4).

Molecular Weight Estimation—We estimated the molecular weights of purified His6-HpaG and His6-HpaG(L50P) using a Superdex-200 HR 10/30 column with an AKTA explorer FPLC system (Amersham Biosciences). The column was equilibrated with 20 mM Tris-HCl (pH 8.0), and the molecular weight of the eluted protein was estimated by a calibration curve prepared using the elution times measured for protein standards (Bio-Rad).

Preparation of XVM2 and Apoplastic Fluid—The hrp induction minimal medium XVM2 (pH 6.7, 20 mM NaCl, 10 mM (NH4)2SO4, 5 mM MgSO4, 1 mM CaCl2, 0.16 mM KH2PO4, 0.32 mM K2HPO4, 0.01 mM FeSO4, 10 mM fructose, 10 mM sucrose, 0.03% casamino acid) was prepared as described previously.
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(29). Apoplastic fluid was obtained from tobacco leaves using gentle centrifugation as described previously (30, 31). Leaves were excised at the base of the petiole with a razor blade, and the petiole was immersed in deionized water. The leaves were then rolled and placed into a plastic syringe. Leaf-filled syringes were centrifuged at 4 °C. The first centrifugation was performed for 15 min at 4,000 rpm, followed by a second centrifugation for 15 min at 10,000 rpm. The second centrifugation was followed by a gentle centrifugation as described previously (30, 31). Leaves then were treated with proteinase K to a final concentration of 100 μg/ml, and the reaction mixtures were brought to 37 °C with agitation. Aliquots were removed at the indicated times and measured on a spectrophotometer (with a 1-mm path length at 27 °C). A scanning rate of 100 nm/min, a time constant of 1 s, a bandwidth of 1 nm, and a resolution of 0.5 nm were used. The spectra of samples containing 4 μM HpaG and its derivatives or 50 μM HpaG peptide and HpaG(L50P) peptide were recorded.

Circular Dichroism Spectroscopy—CD spectra between 190 and 260 nm were measured on a spectropolarimeter (J-715; Jasco, Tokyo, Japan) using quartz cells with a 1-mm path length at 27 °C. A scanning rate of 100 nm/min, a time constant of 1 s, a bandwidth of 1 nm, and a resolution of 0.5 nm were used. The spectra of samples containing 5 μM HpaG and its derivatives or 40 μM HpaG peptide and HpaG(L50P) peptide were recorded.

Congo Red (CR) Binding and Birefringence—A 500 μM stock solution of CR (Sigma) was prepared in 10 mM Tris-HCl (pH 7.6) and 150 mM NaCl. CR binding or red-shift assay was carried out as described previously (27, 33). Mixtures of 10 μM CR and 40 μM His6-HpaG fibrils were incubated at room temperature for 30 min before spectral analysis. Absorbance spectra in the region between 300 and 700 nm were recorded for the His6-HpaG fibril-CR mixture, as well as for CR and His6-HpaG. The corrected His6-HpaG fibril-CR spectrum was obtained by subtracting the spectrum of His6-HpaG alone from the His6-HpaG fibril-CR spectrum (uncorrected). For the apple green birefringence test (34), aliquots (7 μl) of an HpaG fibril suspension (40 μM in 20 mM Tris-HCl (pH 8.0) and 10 mM NaCl) were allowed to dry on a glass slide. The fibrils were stained using a freshly filtered CR solution (0.2% in H2O). Excessive or nonspecific CR staining was removed by washing twice for 1 min with 20% ethanol. Samples were analyzed under a Leica DM RXP light microscope (Leica Microsystems AG, St. Gallen, Switzerland) equipped with a polarizer.

Protease K Resistance Assay—To assess the resistance of aggregated HpaG to protease K, fibrils of His6-HpaG at 40 μM were treated with protease K to a final concentration of 14 μg/ml, and the reaction mixtures were brought to 37 °C with agitation. Aliquots were removed at the indicated times and transferred to SDS-PAGE sample buffer containing 3 mM phenylmethylsulfonyl fluoride and boiled for 3 min. Samples were run on 15% SDS-polyacrylamide gels, followed by staining with Coomassie Brilliant Blue R-250.

Design of Missense Mutations in Motifs 2 and 3—Site-directed mutagenesis of motifs 2 and 3 of HpaG (Fig. 1B) was designed based on the secondary structure of the protein using the HNN secondary structure prediction method (35) and Protscan (DNAstar Lasergene software; DNAstar, Inc., Madison, WI).
Construction of Missense Mutations in Motifs 2 and 3—Site-directed mutagenesis of hpaG was performed using the PCR-mediated megaprimer method (36) to obtain missense mutations in motifs 2 and 3. In the first PCR, pHpaGMT was used as the template DNA with M13–20 and individual mutagenic primers (supplemental Table S2). The PCR conditions and extraction of PCR products from agarose gels were described in a previous report (1). In the second PCR, pJ14 DNA was used as the template, whereas the T7 promoter primer and the megaprimer of the gel elution product from the first PCR were used as primers. The reaction conditions were the same as in the first PCR. The products were purified by phenol and chloroform extraction, and the DNA was precipitated with ethanol. After digestion with NdeI and BamHI, the PCR products were fused to the corresponding sites in the pET14b vector to generate the constructs of His6-HpaG, His6-HpaGΔC66, and His6-HpaGL50P.

**FIGURE 3. EM analysis and HR assay of His6-HpaG, His6-HpaGΔC66, and His6-HpaGL50P.**

**A,** fibril formation of 40 μM His6-HpaG in 20 mM Tris-HCl (pH 8.0) and 10 mM NaCl and its HR inducing activity at various time points. **B,** fibril formation of 5 μM His6-HpaG in XVM2 and its HR inducing activity at various time points. **C,** fibril formation of 5 μM His6-HpaG in apoplastic fluid (AF). **D,** fibril formation of 5 μM His6-HpaGΔC66 and its HR inducing activity in XVM2. **E,** lack of fibril formation of 5 μM His6-HpaGL50P and its lack of HR inducing activity. HR inducing activity was measured using the critical dilution method. Proteins at 10, 5, 2.5, 1, 0.5, and 0.1 μM in 20 mM Tris-HCl (pH 8.0) were injected into tobacco (N. tabacum cv. Samsun NN) leaves, which were photographed 20 h after injection. Time point 0 h is defined as the moment immediately following dilution of the proteins in XVM2 (scale bars = 200 nm).

**FIGURE 4. CD spectroscopic analysis of His6-HpaG and its derivatives.**

**A,** CD spectra of freshly purified 5 μM His6-HpaG and His6-HpaGL50P in XVM2 at 27 °C. **B,** CD spectra of freshly purified 5 μM His6-HpaGΔC66 in XVM2 at 27 °C. The fibrils of His6-HpaG and His6-HpaGΔC66 show the CD spectra of the α-sheet structure (A and B, dashed lines). Time point 0 h is defined as the moment immediately following dilution of the proteins in XVM2.
RESULTS

HpaG Forms Biologically Active Spherical Oligomers—To determine the biochemical characteristics of HpaG, we purified soluble His$_6$-HpaG (15.6 kDa). Freshly purified His$_6$-HpaG separated by size exclusion chromatography showed one major peak at 58.9 kDa, corresponding to a tetramer (Fig. 2A); however, the null HR mutant, His$_{60}$-HpaG(L50P), was purified as a monomer (Fig. 2A). Freshly purified His$_{60}$-HpaG exhibited bead- and annular-like spherical forms similar to the spherical oligomers (amylospheroid) of Aβ protein under EM (Fig. 2B). These forms of HpaG induced an HR, whereas His$_{60}$-HpaG(L50P) did not form bead-like oligomers and failed to induce an HR (Fig. 2, C and D).

Fibril Formation by HpaG—Because purified His$_{60}$-HpaG formed a clear gel after 10 days at 27 °C, we observed the process of fibril formation by His$_{60}$-HpaG daily for 10 days in 20 mM Tris-HCl (pH 8.0) and 10 mM NaCl at 27 °C under EM. The fibrillization of 40 μM His$_{60}$-HpaG proceeded via spherical oligomers and protofibrillar intermediates after 0–8 days of incubation, and curvilinear and partially elongated protofibrils of various sizes were detected after 4–8 days of incubation (Fig. 3A). Mature His$_{60}$-HpaG fibrils were detected after 10 days and appeared as 5–7-nm-wide fibers of various lengths (Fig. 3A).

To study HpaG aggregation under plant apoplast-like conditions, we examined fibril formation in XVM2 and apoplastic fluid. Initially, the concentration of the proteins was lowered to 5 μM, which was the minimum concentration for CD spectroscopic analysis; however, at 5 μM, CD spectroscopy of the HpaG peptide was impossible. Therefore, we increased the concentration of the HpaG peptide to 40 μM His$_{60}$-HpaG(L50P) in 20 mM Tris-HCl (pH 8.0) and 10 mM NaCl (Fig. 3, B and C). Partially elongated fibrils of His$_{60}$-HpaG were detected within 1 day of incubation, and mature fibrils were detected after 3 days of incubation (Fig. 3B), whereas curvilinear forms were detected at 0 h. In apoplastic fluid, His$_{60}$-HpaG formed insoluble precipitates; however, curvilinear forms and fibrils were detected within 1 day of incubation in the insoluble fraction (Fig. 3C). In the case of His$_{60}$-HpaG(L50P), curvilinear and fibrillar forms were observed at 0 h and mature fibrils were detected within 12 h of incubation in XVM2 (Fig. 3D). When the HR inducing activity of the protofibrils and fibrillar forms of His$_{60}$-HpaG and His$_{60}$-HpaG(L50P) was measured at various protein concentrations, both forms were capable of inducing an HR (Fig. 3, A, B, and D). In contrast, the null HR mutant His$_{60}$-HpaG(L50P) did not form mature fibrils (Fig. 3E) and failed to induce an HR (Fig. 3E). These results suggest that the formation of HpaG fibrils under plant apoplast-like conditions is closely related to the HR inducing activity of the protein.

HpaG Forms β-Sheet-rich Fibrils—To determine the transition in the secondary structure of HpaG during oligomerization, we used CD spectroscopic analysis. At 10 h, freshly prepared solutions of 5 μM His$_{60}$-HpaG in XVM2 generated a CD spectrum with minima at 208 and 222 nm, characteristic of a primarily α-helical protein, but the amplitude of positive band in 195 nm was reduced by approximately one-third compared with that for 5 μM His$_{60}$-HpaG in 20 mM Tris-HCl (pH 8.0) containing 10 mM NaCl (Fig. 4A and supplemental Fig. S1). This might have been due to the high absorbance of inorganic salts and other components present in XVM2 at 195 nm. After 3 days, the CD spectrum of HpaG changed to a minimum at 220 nm, which is indicative of transition to a β-sheet (217 ± 5 nm) (Fig. 4A). The CD spectrum of His$_{60}$-HpaG(C66) indicated an α-helix at 0 h, but a β-sheet-rich conformation was observed after 12 h at 27 °C (Fig. 4B), which is consistent with the mature fibrils detected by EM after 12 h incubation (Fig. 3D). Mature fibril formation and transition to a β-sheet from an α-helix by His$_{60}$-HpaG(C66) was much faster than by His$_{60}$-HpaG. A CD spectrum of His$_{60}$-HpaG(L50P) suggested a randomly coiled structure, confirming that the N-terminal α-helical structure is important for HR eliciting activity (Fig. 4A). This was further
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FIGURE 6. CR binding and proteinase K digestion of His6-HpaG fibrils. A, absorbance spectrum of the CR solution in the absence (dashed line) and presence (solid line) of His6-HpaG fibrils, corrected for fibril scattering. The dotted line indicates the difference between CR with HpaG fibrils and CR alone. B, photomicrographs of His6-HpaG fibrils stained with CR under bright field and polarized light. C, proteinase K (PK) resistance assay. SDS-PAGE of soluble (upper panel) and aggregated (lower panel) His6-HpaG treated with 14 μg/ml proteinase K. Θ denotes the sample without proteinase K, and the incubation times for the proteinase K digestion were 0, 1, 2, 5, 10, 15, and 20 min. M denotes protein molecular markers.

corroborated by the CD spectra of HpaG peptide (H2N-NQGISEKQLDQLTTQILMLLQQ-COOH) and HpaG(L50P) peptide (H2N-NQGISEKQLDQLTTQILMAPLQQ-COOH). HpaG peptide showed a typical α-helical structure, whereas HpaG(L50P) peptide showed a randomly coiled structure (Fig. 5A). The observation of partially elongated fibrils of the HpaG peptide and the loss of HR inducing activity and fibril-forming ability of the HpaG(L50P) peptide (Fig. 5B) indicate that the N-terminal α-helix motif of HpaG is critical for fibril formation and harpin function.

HpaG Is an Amyloid Protein—To determine whether the fibrillar form of HpaG is amyloid, we performed a binding assay with CR. Like other amyloid proteins, His6-HpaG fibrils retained the ability to bind CR, causing a red shift in the spectrum with a maximum difference in absorbance between CR with HpaG fibrils and CR alone. In the absence (motif 2 (HpaG-C1 and HpaG-C2) and motif 3 (HpaG(L93P), HpaG(L101P), and HpaG(L93P/L101P)) (Fig. 1B). The HR inducing activity of these mutants was the same as that of wild-type HpaG (Fig. 1B); however, those proteins with missense mutations in motif 3 showed more rapid fibril formation in XVM2 than wild-type protein (Figs. 3B and 7). In the case of the motif 2 missense mutations, HpaG-C1 showed the same fibril formation rate as wild-type HpaG (Fig. 7), whereas HpaG-C2 formed fibrils faster than HpaG-C1 but slower than the motif 3 missense mutants (Fig. 7). These results suggest that motif 3 affects the rate of His6-HpaG fibril formation.

Other Harpins Form Fibrils—To determine whether fibril formation is a common feature of harpins, we performed in vitro fibrillogenesis assays with His6-XopA from Xanthomonas campestris pv. vesicatoria, His6-HrpN from E. amylovora, and His6-HrpZ from P. syringae pv. syringae. As previously reported, XopA is an HpaG homolog but lacks the ability to induce an HR in tobacco. Wild-type His6-XopA did not form fibrillar structures; however, the gain-of-function mutant His6-XopA(F48L/M52L) was able to form curvilinear protofibrils and fibrils in XVM2 (Fig. 8A), suggesting a positive correlation between fibrillogenesis and HR elicitor activity. Interestingly, HrpN and HrpZ at 5 μM exhibited fibrillar and curvilinear structures, respectively, after 1 day of incubation in XVM2 (Fig. 8B).

DISCUSSION

Although harpins were discovered more than a decade ago (3, 9–13), their biochemical role and the mechanism by which they cause HRs and affect the pathogenicity of plant bacteria remain largely undetermined. They have been classified as helpers, not as effectors, in hrp type III protein secretion systems. This is because of the site of function of harpins in plant cells and, in some cases, the negligible role of harpins in pathogenicity (2, 37). Harpins function in intercellular spaces and are known to possess pore-forming activity (16–18). It is noteworthy that the prominent feature of harpins is a high glycine content that is involved in bonding with hydrophobic membranes (38); however, the biochemical basis of plant cell membrane destabilization by harpins is unknown.

We demonstrated that His6-HpaG forms a tetramer when it is overexpressed in Escherichia coli, which was the first demonstration of a basic biochemical unit for a harpin. Because the null HR mutant HpaG(L50P) failed to form a tetramer, it is presumed that the Leu-50 residue in the primary sequence of HpaG plays a role in oligomerization. Moreover, the leucine residue at this position is conserved among active harpins (4). Considering the low molecular weight (15.6 kDa) of mono-
meric His$_6$-HpaG, we did not expect to see spherical particles under EM. Furthermore, the spherical particles did not seem to correspond to the molecular weight of the tetramer, which may have occurred because of further oligomerization of His$_6$-HpaG that may have occurred during EM preparation. The fact that His$_6$-HpaG and A$_{40}$ form spherical oligomers suggests that both proteins have common biochemical features.

We previously showed that HpaG has $\alpha$-helical features important for its ability to induce HRs, and that $\alpha$-helices are predicted in the N-terminal regions of several harpins (4). This implies that the N-terminal $\alpha$-helix (motif 1) is a key functional domain for harpin activity. In addition, the N-terminal $\alpha$-helical region has a high leucine content (26%), which is consistent with previous observations that leucine is a helix-stabilizing residue often found at the N terminus of $\alpha$-helical antimicrobial peptides, where it plays an important role in stabilizing the membrane-bound protein structure (38, 39). This study confirms for the first time that the predicted N-terminal $\alpha$-helix of bacterial harpin HpaG actually forms an $\alpha$-helix, and that this formation is important for its HR inducing activity. Because the presence of an $\alpha$-helix was predicted for HrpZ of P. syringae pv. syringae (40), $\alpha$-helices in harpins might play common roles. Our CD spectral analysis of His$_6$-HpaG and the mutant form His$_6$-HpaG(L50P) validates this hypothesis.

The misfolding and aggregation of proteins into fibrillar amyloid are important features of several diseases affecting humans and animals (20, 21), and in vitro amyloid fibril formation proceeds via soluble oligomers or protofibrillar intermediates that eventually form more stable $\beta$-sheet-rich amyloid fibrils (23, 41). We observed a “curvilinear form” of His$_6$-HpaG at $t = 0$ in XVM2 or in apoplastic fluid, indicating that protofibril formation occurs immediately and that a curvilinear form of His$_6$-HpaG is biologically active. In Alzheimer disease, however, an amylospheroid form of A$_{40}$ is known to be more neurotoxic than the fibrillar form (42). When we observed the process of fibril formation for HpaG, amyloid fibril formation proceeded via spherical oligomers and protofibrillar intermediates, such as curvilinear structures (43). This indicates that HpaG undergoes a process of fibril formation that is similar to A$_{40}$; however, unlike A$_{40}$ fibrils in Alzheimer disease, the protofibrils and mature fibrils of HpaG induced HRs of equal severity. This was unexpected because small prefibrillar oligomers and other intermediates in amyloid formation are believed to be responsible for cell death, whereas mature fibrils are thought to be inert misfolded protein end products (44, 45).

The fact that His$_6$-HpaG, His$_6$-HrpN, and His$_6$-HrpZ form protofibrils and/or fibrils under apoplast-like conditions within
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1 day is in good agreement with the time necessary to observe an HR when the protein is infiltrated into tobacco leaves. Therefore, it is very likely that amyloidogenesis of HpaG occurs in vivo and is important for its HR inducing activity. Unfortunately, a study of His$_6$-HpaG in apoplastic fluid could not be performed because the tagged protein was insoluble, and many unknown components, including various proteins and salts, in the apoplastic fluid impeded CD spectroscopic analysis.

Because the null HR mutant HpaG(L50P) showed a randomly coiled structure using CD spectroscopy, motif 3 may not form an α-helical structure; however, we found that motif 3 modulates the rate of HpaG fibril formation, suggesting a possible intramolecular interaction between motifs 1 and 3. A similar phenomenon has been observed in the yeast prion URE3, which exhibits intramolecular interactions between the N- and C-terminal domains (46). The motif 2 region, which is predicted to be flexible, may play an important role in the intramolecular interaction between motifs 1 and 3 during HpaG fibril formation; however, the function of motif 2 in HpaG is still unclear because HpaGΔC75, which lacks motif 2, possesses harpin properties and undergoes amyloid fibril formation.

Aβ protein, α-synuclein, and prion proteins are known to promote the formation of distinct ion-permeable, pore-forming protofibrils that cause cell membrane breakage (47, 48). Harpins also have membrane pore-forming and destabilizing activities (16–19). Therefore, it is plausible that His$_6$-HpaG might destabilize membranes like other harpins. This possibility is currently under investigation.

HpaG is unique, because amyloid-forming proteins are found in several microorganisms (14, 24–26), but they are not cytotoxic, except for microcin E492 (Mcc) (19). HpaG amyloid and previously reported bacterial amyloids suggest that additional bacterial proteins possess fibrillar aggregation properties. Thus, an amyloid may be a generic structural form of protein, but a bacterial amyloid may be the product of specific functional changes of a protein, rather than simply being an inert end product. Indeed, curli fibrils in E. coli serve structural functions (27); Mcc fibrils have a modulatory effect on biological activity (19), and HpaG fibrils induce HRs.

In conclusion, the data presented in this study contribute to our understanding of the important role of harpins in plant cell death, and the data suggest possible common biochemical mechanisms of rapid plant death and animal cell death caused by disease-related amyloid proteins. Understanding the involvement of amyloids in the interaction of plants with plant pathogenic bacteria will provide new insights into the nature of fibril-forming proteins as well as into the mechanisms of cell death because of amyloid-forming proteins.

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