LEUCINE ZIPPER-LIKE MOTIFS OF HRPZPSS ARE NOT ESSENTIAL TO INDUCE HYPERSENSITIVE RESPONSE IN TOBACCO

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SUMMARY

Harpins are heat-stable, glycine-rich proteins secreted by several Gram-negative phytopathogenic bacteria, involved in the induction of hypersensitive response in non-host plants. HrpZPss is highly heat stable, exists as a polydispersed, multimeric protein in nature and the functional significance for its existence in many oligomeric forms, remains still unclear. It was suggested that the leucine zipper-like motifs (LZMs) may take part in the formation of oligomeric aggregates, which may be responsible for HR elicitation by harpins and for their high thermal stability. To test the involvement of LZMs in HR elicitation and induction of defense responses, we have generated deletion mutants with or without LZMs and analysed their ability to induce HR, and/or activate the defense responses. The deletion mutant HrpZMM1, with no LZM also elicited HR as well as defense responses in tobacco suggesting the LZMs are not essential for elicitor activity of HrpZPss. However, the ability to elicit defense responses may be linked with the ability to induce HR.

Key words: HrpZPss, Hypersensitive response, truncated mutations.

INTRODUCTION

Harpins are proteinaceous elicitors of defense and hypersensitive response (HR) in a wide range of crop plants, produced by plant pathogenic bacteria like Pseudomonas, Erwinia, Xanthomonas and Ralstonia. HrpZPss from Pseudomonas syringae pv. syringae retains its biological activity in several deletion mutants, at different sites, (Alfano et al., 1996) in an unique manner. The activation of different responses by harpin was assigned to different structural features. Intact HrpZpph is required for the formation of ion-conducting pores, whereas for the induction of defense responses, only its C-terminal part is crucial (Engelhardt et al., 2009). Sequence analysis of HrpZPss revealed the presence of at least two possible leucine-zipper-like motifs (LZMs) which were also found in other harpins (Tarafdar et al., 2009). LZMs are located on helices present on protein surfaces, signifying that they can take part in the formation of oligomeric aggregates, which may be responsible for HR elicitation by harpins and for their high thermal stability (Tarafdar et al., 2009). Random and site directed mutagenesis of HpaG, from Xanthomonas axonopodis pv. glycines enabled the identification of a region essential for elicitor activity, consisting of a peptide 23 amino acid (aa) in size (H2N-NQGISEKQLDQLLTQLIMALLQQ-COOH) (Kim et al., 2004). This region shared a homology of 78% and 74% with 23 and 27 aa regions of the HrpW of Pseudomonas and Erwinia spp, respectively. The stretch of 12 highly hydrophilic aa (H2N-QGISEKQLDQLLTQLIMALLQQ-COOH) that partially overlaps the N-terminal α-helical regions of the respective proteins is critical for the elicitation of HR in tobacco by Hpa1Xoo and Hpa1Xoc. Further, the coiled coil integrity of the harpin is important for dimerization and HR elicitation in tobacco as revealed by two single missense mutants Hpa1Xoo (L51P) and Hpa1Xoc (L53P) (Wang et al., 2008). Although these aa are crucial for elicitation of HR in tobacco, they are not necessarily involved in the induction of systemic acquired resistance against Tobacco mosaic virus (TMV) (Wang et al., 2008), which reveals that the dual roles of harpin require different structural determinants.

HrpZPss has a single tryptophan (W167) residue, which makes it a protein of special interest for fluorescence quenching studies. HrpZPss sequence analysis revealed its dissimilarity in aa sequence (He et al., 1993; Engelhardt et al., 2009) and lack of homology with any known protein in databases, which makes it a unique biological molecule for functional or structural studies. Although HrpZPss was biochemically characterized, and also analyzed by mutational analysis, its high thermal stability was not addressed from a structural point of view. Circular dichroism (CD) analysis revealed that the protein is predominantly α-helical (51.5%) and contains a high percentage (13.5%) of leucine (He et al., 1993). The primary structure of HrpZPss contains heptad repeats of leucine and other hydrophobic aa (leucine zipper-like motif; LZM). Two possible LZMs were found in the region of 73-87 (219-261 base pairs, bp) and 245-266 (735-798 bp) aa stretch of HrpZPss. Comparable leucine-zipper-like motifs were also predicted in eight other harpins (Tarafdar et al., 2009) and one more possible heptad LZM was identified in the 299-334 (897-1002 bp) aa stretch. LZMs in most of the harpins are composed by...
coiled-coil motifs, where the first and fourth positions of the heptad repeats are occupied by hydrophobic aa (Tarafdar et al., 2009). LZMs are thought to be responsible for the dimeric or oligomeric forms for some α-helical proteins (Landschulz et al., 1988). Therefore, as reported in the present study, we designed various truncations of HrpZPs and expressed the truncated peptides to analyze the role of LZMs in inducing defense responses or elicitation of HR.

**MATERIALS AND METHODS**

Cloning, expression and purification of HrpZPs and its truncated mutants. DNA encoding hrpZps (GenBank ID: L14775) of *Pseudomonas syringae* pv. *syringae* for the five truncated genes and one intact gene were amplified on hrpZps-pYEUT (Podile et al., 2001) template at 60°C using Pfu DNA polymerase. Primers (Table 1) were designed to amplify 360 bp of C-terminal (C1), 255 bp of C-terminal (C2), 327 bp of N-terminal (N1), 386-810 bp (MM1), 386-810 bp (MM2) and intact hrpZps incorporating NdeI and XhoI sites for directional cloning into pET28a. The details of the possible LZMs in different HrpZPs mutants are given in Table 2. Purified PCR products and pET28a were double digested, gel extracted and ligated. Escherichia coli DH5α competent cells were transformed with ligation mixtures individually. Colonies were screened for recombinant plasmids by colony PCR, double digestion and sequenced to verify the accuracy of the sequence and reading frame.

Confirmed plasmids were mobilized into expression host *E. coli* BL21-DE3 for protein expression as described previously (Tarafdar et al., 2009). Overnight culture was diluted 50 times with fresh LB broth with antibiotics (Tarafdar et al., 2009) and induced with 100 μM IPTG at OD600 0.4. Cell pellet was collected by centrifugation at 8,000 g, washed and re-suspended in 10 mM sodium phosphate buffer (pH 6.8) containing 50 mM NaCl. Soluble proteins were extracted by sonication and centrifugation at 10,000 g. A protease inhibitor cocktail (S8830, Sigma-Aldrich, USA) was added to minimize the degradation during sonication. Soluble supernatant was subjected to Ni-NTA affinity chromatography using HIS-Select Nickel Affinity Gel (P6611, Sigma-Aldrich, USA). His-tagged proteins were eluted using Imidazole gradient and quantified by protein estimation using the Bradford reagent (Bradford, 1976). Fractions containing high amount of protein were pooled and resolved on 15% SDS-PAGE. Each fraction was probed with α-helical antibody (Abcam, USA).

**HR inducing ability of HrpZPs peptides.** The HR inducing ability of HrpZPs peptides was evaluated in five to six-leaf stage greenhouse-grown *Nicotiana tabacum* cv. Xanthi leaves. HrpZPs peptides (20 µM) in phosphate-buffered saline (PBS) were infiltrated using a hypodermic syringe. Infiltration with EVP served as negative control. The plants were maintained at 27±2°C with 14h/10h day and night conditions for 36 h to allow the HR to develop.

**Induction of defense gene transcripts by HrpZPs peptides.** Five-leaf stage tobacco plants were infiltrated (at least three leaves per protein with two spots per leaf) with HrpZPs peptides (20 µM) and EVP (mock infiltration). After 12 h, a whole leaf was collected (ca. 1.5 g), frozen in liquid nitrogen and ground to fine powder. Total RNA was extracted using TRI reagent (Sigma-Aldrich, USA) and analyzed by cDNA synthesis and reverse transcription (RT) PCR. The list of primers used is given in Table 1.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ to 3’</th>
<th>Target</th>
</tr>
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<tbody>
<tr>
<td>HrpFP</td>
<td>CGAATTCATATGCAAGATCAGTGCTCAGTCTTAAC</td>
<td>HrpZPsw</td>
</tr>
<tr>
<td>HrpRP</td>
<td>CGGGGATCCCTGAGGGCTGCAGCTGCAATTGC</td>
<td>HrpZCI</td>
</tr>
<tr>
<td>HrpC1FP</td>
<td>CGAATTCATATGACAACTCGTCCGTGATG</td>
<td>HrpZC2</td>
</tr>
<tr>
<td>HrpC2FP</td>
<td>CGAATTCATATGGACCGTGGCCTGCAATTGC</td>
<td>HrpZNI</td>
</tr>
<tr>
<td>HrpMM1FP</td>
<td>CGAATTCATATGAACTCGTCCGTGACACTTC</td>
<td>HrpZMM1</td>
</tr>
<tr>
<td>HrpMM1RP</td>
<td>CCGGATCCCTGAGGTCTGACCTGCACGGGACC</td>
<td>HrpZMM2</td>
</tr>
<tr>
<td>HrpMM2FP</td>
<td>CCGGATCCCTGAGGTCTGACCTGCACGGGACC</td>
<td>N. tabacum pal</td>
</tr>
<tr>
<td>HrpMM2RP</td>
<td>CCGGATCCCTGAGGTCTGACCTGCACGGGACC</td>
<td>N. tabacum PR1a</td>
</tr>
<tr>
<td>Nt-PALFP</td>
<td>CCGGATCCCTAGGCAGAGATGAGTAGGCTGCTCAGTCTTAAC</td>
<td>N. tabacum hin1</td>
</tr>
<tr>
<td>Nt-PALRP</td>
<td>TCGGATCCCTGAGGTCTGACCTGCACGGGACC</td>
<td>N. tabacum hsr203J</td>
</tr>
<tr>
<td>Nt-PR1aFP</td>
<td>AGCTGTCGAGATGAGTAGGCTGCTCAGTCTTAAC</td>
<td>N. tabacum hsr203J</td>
</tr>
</tbody>
</table>
cDNA was synthesized by RT-PCR using BluePrint 1st Strand cDNA synthesis kit (Takara Bio, Japan). An equal amount of 4 μg of total RNA from each treatment was used for cDNA synthesis and 1 μl of the synthesized cDNA mixture was used as template for test transcript amplification. Primers were designed (Table 1) for four selected defense gene transcripts (PAL, PR-1, HIN1 and HSR203J) to give 350-400 bp amplicon. PCR was performed at 60°C for 30 cycles, to amplify the selected transcripts using cDNA, synthesized from HrpZPss peptides-treated leaves, as a template.

**Defense-responses induced by truncated HrpZPss mutants.** To characterize defense-responses induced by truncated HrpZPss mutants, sub-lethal concentration (1 μM) of intact and truncated mutants of HrpZPss were used. Leaf samples were collected after 18 h (standardized from a series of 12- 24 h) of infiltration for all the biochemical assays. Thiobarbituric acid reactive substances (TBARS) were measured as described by Rustérucci et al. (1996). Ascorbate peroxidase (EC 1.11.1.11 - L-ascorbate peroxidase) was assayed as described by Nakano and Asada (1981). Soluble proteins were extracted in 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H₂O₂. The activity was measured as decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated. For collagen staining, leaf pieces were boiled in 1 M KOH until they turned to light yellow, washed several times with deionized water (Hood and Shew, 1996). The processed leaf samples, stained with 0.05% aniline blue (in K₂HPO₄; pH 9.0), were observed under confocal laser scanning microscope (Leica, Germany).

**Table 2.** Details of leucine zipper-like motifs present in different truncated mutants of HrpZPss. Boxes indicate the location of the leucine zipper-like motifs in the respective mutants.

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Sequence region</th>
<th>Description</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HrpZPss</td>
<td>1-1021</td>
<td>Three LZMs with intact sequence</td>
<td></td>
</tr>
<tr>
<td>HrpZC1</td>
<td>661-1021</td>
<td>2 C-terminal LZMs with missing N-terminal portion of 660 bp</td>
<td></td>
</tr>
<tr>
<td>HrpZC2</td>
<td>766-1021</td>
<td>1 C-terminal LZMs with missing N-terminal portion of 765 bp</td>
<td></td>
</tr>
<tr>
<td>HrpZN1</td>
<td>1-327</td>
<td>One N-terminal LZM with missing C-terminal portion of 693 bp</td>
<td></td>
</tr>
<tr>
<td>HrpZMM1</td>
<td>271-720</td>
<td>No LZM with missing 270 bp N-terminal region and 300 bp C-terminal region.</td>
<td></td>
</tr>
<tr>
<td>HrpZMM2</td>
<td>386-810</td>
<td>One N-terminal LZM with missing N-terminal portion of 385 bp and 210 bp of C-terminal region.</td>
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**RESULTS**

**Generation, expression and purification of HrpZPss and truncation mutants.** Two N-terminal deletions hrpZC1 and hrpZC2 (with two and one C-terminal LZM sequences respectively), eliminating 660 and 765 bp and one C-terminal deletion hrpZN1 (with one N-terminal LZM sequence) eliminating 693 bp, were generated in a PCR-based approach by introducing the start and stop codons in to the primer sequence. Two additional mutants were generated by eliminating small portions at both the ends; hrpZMM1 and hrpZMM2 (without any LZMs and with one middle LZM, respectively). Amplicons size was compared against a DNA marker along with intact hrpZPss. The amplicons were 1020, 360, 255, 327, 449 and 425 bp, respectively, for intact hrpZPss, hrpZC1, hrpZC2, hrpZN1, hrpZMM1 and hrpZMM2. The six amplicons (including the intact hrpZPss and the five truncated versions of hrpZPss) were separately cloned in to the Ndel and XhoI sites of pET28a. The resultant plasmids were designated as pETZ, pETC1, pETC2, pETN1, pETMM1 and pETMM2, respectively, for hrpZ, hrpZC1, hrpZC2, hrpZN1, hrpZMM1 and hrpZMM2.

The six versions of harpinPss (HrpZ, HrpZC1, HrpZC2, HrpZN1, HrpZMM1 and HrpZMM2) were expressed in *E. coli* BL21(DE3) and resolved on 15% SDS PAGE. Molecular mass of the harpin peptides was 38, 14, 10, 12, 17 and 16 kDa (with the hexa-histidine tag), respectively, for full-length HrpZ, HrpZC1, HrpZC2, HrpZN1, HrpZMM1 and HrpZMM2 (Fig. 1).

**HR inducing ability of HrpZPss peptides.** Five truncated mutants (HrpZC1, HrpZC2, HrpZN1, HrpZMM1
and HrpZM2) and the intact HrpZPss were infiltrated into tobacco to induce HR. Withering of infiltrated area was visible 12 h after infiltration that culminated into 100% HR by 24 h in all mutant HrpZPss-infiltrated areas. The HR induced by mutants was faster, (visible during 12-18 h) than that of HR induced by intact HrpZPss, and the difference slowly disappeared by 24 h (Fig. 2a). No significant difference was visible in HR-inducing ability among the five truncated mutants, with respect to the time of appearance and intensity of the HR. The infiltrated area collapsed completely after 24 h. The mutant HrpZMM1, without any LZM also elicited HR, suggesting no role for LZM in HR induction. There was no effect of deletion of a particular portion on either termini (N- or C-) in the HR-inducing ability of HrpZPss.

**Induction of defense genes by HrpZPss peptides.** Treatment with truncated mutants resulted in an increase of transcripts of defense and HR markers in tobacco. All the five truncated mutants (HrpZC1, HrpZC2, HrpZN1, HrpZMM1 and HrpZMM2) altered the transcript level of the marker genes similarly to that of intact HrpZPss (Fig. 2b). There was no effect of deletion of the LZMs on the
ability of the HrpZ<sub>Pss</sub> in activation of defense genes in tobacco.

**Defense responses induced by HrpZ<sub>Pss</sub> peptides.** The HR induced by truncated mutants of HrpZ<sub>Pss</sub> was compared by measuring lipid peroxidation, ascorbate peroxidase and accumulation of callose. The TBARS in truncated HrpZ<sub>Pss</sub> protein(s)-treated tobacco leaves increased at least two-folds within 18 h in all the treatments (Fig. 3a). A nearly two-fold increase of ascorbate peroxidase was observed in all mutant HrpZ<sub>Pss</sub>-treated leaves compared to EVP treatment (Fig. 3b).

All truncated HrpZ<sub>Pss</sub> mutants showed indistinguishable ability to induce callose deposition in treated tobacco leaves within 18 h (Fig. 4). The pattern of callose deposition was similar among the mutants and between mutants and intact HrpZ<sub>Pss</sub>. No significant role was observed for the LZMs of HrpZ<sub>Pss</sub> in altering/inducing defense responses in tobacco.

**DISCUSSION**

HrpZ causes HR and induces resistance in non-host plants. Initially, the presence of repeats of specific aa like QTGT and GGGLGGTP was thought to be responsible for HR-eliciting activity of HrpZ<sub>Pss</sub> (He et al., 1993). Later, it was suggested that the biological activity was independent of these sequences (Alfano et al., 1996). More than one region in the entire sequence was responsible for the induction of HR by HrpZ<sub>Pss</sub> and a few smaller fragments induced stronger HR in tobacco (Jin et al., 1997). It is uncommon that any small portion of a protein retains its total biological activity. Since the non-overlapping HrpZ peptides cause HR in non-host tobacco (He et al., 1993) and cell death in S. cerevisiae (Sripriya, 2004), it was suggested that the activity of HrpZ<sub>Pss</sub> may not be confined to a single region of the protein. Jin et al. (1997) proposed that the internal 10-12 kDa-sized segment may be responsible for induction of stronger HR. Partially digested HrpZ<sub>Pss</sub> induced stronger HR than intact HrpZ<sub>Pss</sub> possibly due to the formation of multiple fragments with same biological activity. It was also possible that, the harpin fragments that are recognized by plant defense-related receptors are more exposed, in contrast with the folded full-length protein which forms oligomers (Taraifar et al., 2009; Haapalainen et al., 2011). This suggests that the active site, which can induce stronger HR may be present at multiple sites of the protein. HrpZ<sub>Pss</sub>D125, which induces stronger HR was a deletion mutant (He et al., 1993). However, partial digestion of this harpin peptide resulted in no HR, indicating that the HrpZ<sub>Pss</sub> peptides smaller than D125 may not be sufficient to induce HR. When the harpin protein is secreted in the plant apoplast, protease digestion may release the different active fragments that can induce HR, similar to the 28 aa peptide produced from avr9 of Cladosporium fulvum (Van Den Ackerveken et al., 1993). However, the proteolytic processing of harpin in minimal medium by Pseudomonas was not confirmed (He et al., 1993).

Here, we have generated six (three N-terminal deletions, one C-terminal deletion, two mutants having deletions at both termini) versions of HrpZ<sub>Pss</sub> with different number(s) of possible LZMs and evaluated the role of LZMs in inducing HR as well as defense responses in tobacco. The mutant HrpZMM1, without any LZM, also induced defense responses, and elicited HR as strongly as intact HrpZ. All six mutants with different sequence lengths, showed no difference in HR-inducing ability in tobacco in comparison with that of intact HrpZ<sub>Pss</sub> (Fig. 2a). We also observed that the mutants triggered a rapid HR compared to the intact HrpZ. This could be due to the molar differences between the high molecular weight of intact HrpZ and the lower molecular weight mutants. Further confirmation was obtained through RT-PCR and biochemical characterization of HR. There was no difference in induction of defense and HR-related responses (Fig. 4) between the mutants with LZMs.

Elicitation of defense responses by truncated mutants of harpin was known. Previous studies indicated that either N-terminal or C-terminal portion of the protein was crucial for the induction of HR as well as disease resistance. We now report that the LZMs are not involved in induction of HR and defense responses by HrpZ<sub>Pss</sub>.

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