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Short Communication

Biological Activities of Purified Harpin$_{Xoo}$ and Harpin$_{Xoo}$ Detection in Transgenic Plants Using Its Polyclonal Antibody

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Abstract Many harpins have been found in plant pathogen bacteria that can elicit disease and insect resistance in plants, and promote plant growth. In this work, we overexpressed and purified *Xanthomonas oryzae* pv. *oryzae* harpin, harpin$_{Xoo}$, in *Escherichia coli* BL21/pGEX-hpa1. Harpin$_{Xoo}$ was fused to the C-terminus of glutathione S-transferase (GST) and purified using the Bulk GST purification module and thrombin cleavage capture kit. Purified harpin$_{Xoo}$ protein was sensitive to protease K and stable to heat treatment, and could not induce a hypersensitive response after treatment with various plant metabolic inhibitors; these characteristics were similar to harpin$_{Ea}$ of *Erwinia amylovora*. The purified harpin$_{Xoo}$ showed a similar ability to induce tobacco mosaic virus resistance in tobacco as harpin$_{Ea}$. Its antibody worked well in detecting the purified harpin$_{Xoo}$, harpin$_{Xoo}$ in the total protein of *E. coli* BL21/pGEX-hpa1 and an *hpa1* transgenic rice.

Key words harpin$_{Xoo}$; glutathione S-transferase (GST); biological activity; polyclonal antibody

Harpin is a group of glycine-rich, heat-stable and protease K-sensitive proteins that are able to elicit disease and insect resistance in plants, induce many plant-reaction phenotypes, and promote plant growth, yield and quality [1–6]. The first harpin, encoded by the gene *hrpN*, was isolated from *Erwinia amylovora*, and named harpin$_{Ea}$ [1]. To date, harpins from four genera of bacterial plant pathogens, *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Ralstonia*, have been reported [7–14].

Plant-pathogen interactions can be classified into two types: compatible and incompatible. In a compatible interaction, the host plant does not mount an effective defense response, and the pathogen causes disease; in an incompatible interaction, the resistant variety or non-host plant effectively prevents the invasion and spread of the pathogen by initiating defense responses or its pre-formed barriers and compounds. Hypersensitive response (HR) is an important characteristic in the defense mechanisms of some incompatible interactions. HR is a localized plant cell death at the site of pathogen infection. The dead cell surrounding the pathogen forms a physical barrier to the invasion of the pathogen. In addition, compounds released from the dead cells may be toxic to the invading pathogen [15]. Earlier research indicates that phytopathogenic bacteria are likely to have the same factors that are responsible for triggering HR in non-host plants and are required for pathogenicity in host plants [16]. Harpin can induce HR in non-host plants, but the role of harpin in sensitive hosts is not clear [17]. Harpins were identified and isolated to study how plant pathogenic bacteria interact with host and non-host plants. Harpins are encoded by hypersensitive response and pathogenicity gene (*hrp*) clusters of plant bacteria [18].

In *Xanthomonas oryzae* pv. *oryzae* (Xoo), a Gram-negative bacterium causing bacterial leaf blight on rice, the *hrp* gene cluster was dissected. The protein was given the name harpin$_{Xoo}$ and the corresponding gene designated *hpa1* (GenBank accession No. AB045311) [19]. *hpa1* was previously expressed in *Escherichia coli* in our laboratory, but harpin$_{Xoo}$ was not purified [11,20]. The purification of harpin$_{Xoo}$ is indispensable to the study of its 3-D structure and the preparation of anti-harpin$_{Xoo}$ polyclonal antibody.

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The glutathione S-transferase (GST) gene fusion system is a simple and fast technique to express and purify proteins [21]. This technique needs few apparatus and obtains abundant purified protein in two days.

In this work, we purified harpin$_{Xoo}$ using the GST gene fusion system, studied its characteristics, and reported its detection in transgenic rice plants by polyclonal antibody.

Experimental Procedures

The gene *hpa1* was amplified from *X. oryzae* pv. *oryzae* strain JxoIII genomic DNA by a standard polymerase chain reaction (PCR). The primers were 5′-TTC*GGATCC*ATGAATTCTTTGAACACACAATT-3′ (forward, *Bam*HI site is in italic) and 5′-GGT*GAATTC*TTACTGCATCGATGCGCT-3′ (reverse, *Eco*RI site is in italic). PCR amplifications were performed for one cycle of 5 min at 95 °C; 35 cycles of 45 s at 95 ºC, 45 s at 56 ºC, 45 s at 72 ºC; and a final extension step of 7 min at 72 ºC. The PCR product was cloned into pGEM-T easy vector (Promega, Madison, USA), a high copy T vector. The positive insertion was confirmed by the automated DNA sequencing in TaKaRa (Dalian, China). The recombinant vector containing *hpa1* (pGEM-hpa1) was digested with *Bam*HI and *Eco*RI, then ligated into pGEX-2T vector (Pharmacia, Uppsala, Sweden) digested with the same enzymes. The recombinant plasmid pGEX-hpa1 was transformed into *E. coli* BL21(DE3) (Pharmacia), and the resulting strain was named *E. coli* BL21/pGEX-hpa1.

Ten microliters of glycerol stock of *E. coli* BL21/pGEX-hpa1 was inoculated into 1 ml of 2×YT medium supplemented with 100 μg/ml ampicillin. After 6 h incubation at 37 ºC, 100 μl culture was inoculated into 10 ml of 2×YT medium and incubated overnight at 37 ºC under the same conditions. Thereafter, the culture was diluted at 1:100 in fresh 2×YT medium with 100 μg/ml ampicillin and incubated at 37 ºC. When the A_{600} reached 0.6–0.8, 0.1 mM isopropyl-β-*D*-1-thiogalactopyranoside (IPTG) was added, and the mixture was incubated for additional 4 h at 28 ºC or 37 ºC to induce the protein expression.

The bacterial cells were harvested and sonicated, and the glutathione Sepharose 4B was prepared according to the instructions of the Bulk GST purification module (Pharmacia). Glutathione Sepharose 4B was added to the cell supernatant, then mixed gently for 30 min at room temperature. The mixture was loaded onto a column provided in the Bulk GST purification module. Harpin$_{Xoo}$ was cut from the GST-harpin$_{Xoo}$ fusion protein and eluted according to the instructions of the Thrombin cleavage capture kit (Novagen, San Diego, USA). Harpin$_{Xoo}$ was then subjected to a boiling water bath for 10 min and centrifuged at 10,000 *g* for 5 min to remove insoluble materials. The supernatant contained purified harpin$_{Xoo}$. The GST was eluted according to the instructions of the manufacturer. The concentration of purified harpin$_{Xoo}$ was measured using the BAC-100 protein quantitative analysis kit (Biocolor Biotech, Shanghai, China).

New Zealand white rabbits (College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China) were immunized subcutaneously at multiple sites with 200 μg of purified harpin$_{Xoo}$ in 0.5 ml of isotonic saline emulsified with an equal volume of Freund complete adjuvant. Animals were boosted at weekly intervals with 30 μg of antigen emulsified with Freund incomplete adjuvant. Six weeks later, the rabbits were bled and the sera were tested for antibody by immunoblotting. Anti-harpin$_{Xoo}$ serum titer was determined by enzyme-linked immunosorbent assay (ELISA) method. Pre-immune sera were also obtained and analyzed in parallel. IgG was isolated from anti-harpin$_{Xoo}$ serum using 40% saturated ammonium sulfate according to an ammonium sulfate precipitation protocol [22]. Anti-harpin$_{Xoo}$ antibodies precipitated from 1 ml of anti-harpin$_{Xoo}$ serum were redissolved in the same volume of phosphate-buffered saline (PBS) and dialyzed overnight against the same buffer.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 13% slab gel according to the method of Sambrook *et al*. [23] and samples were boiled for 5 min before loading. Proteins on the polyacrylamide gel were stained with 0.25% Coomassie brilliant blue R-250 (Amresco, Ohio, USA). In immunoblotting, the heat-treated proteins separated by SDS-PAGE were electrotransferred to a nitrocellulose membrane. The membrane was reacted with polyclonal antibody against harpin$_{Xoo}$ and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Sino-American Biotech, Luoyang, China). Finally the membrane was detected with 3,3′-diaminobenzidine tetrahydrochloride.

The sensitivity to protease K and heat-stability of purified harpin$_{Xoo}$ were measured according to Wen and Wang [11], and the commercial product Messenger containing 3% harpin$_{Ea}$, kindly provided by Dr. Zhong-Min WEI (Eden Bioscience, Annapolis, USA), was used as a control. In brief, protease K or protease K+phenyl methyl sulphonyl fluoride (PMSF, a protease inhibitor, final concentration 0.5 mM) was added to harpin (10 μg/ml) in PBS with final concentration of protease K being 0.2 μg/ml and maintained at 37 °C for 15 min; heat-stability assay was performed

with a boiling water bath for 10 min. Harpins were treated with eucaryotic metabolic inhibitors, cycloheximide, actinomycin D and lanthanum chloride, according to He *et al.* [24].

To induce HR, harpin$_{Xoo}$ and harpin$_{Ea}$, which were treated with boiling water, cycloheximide, actinomycin, lanthanum chloride, protease K, or protease K+PMSF, were infiltrated into *Nicotiana tabacum* L. cv. Xanthi tobacco leaves, the seeds of which were from the Tobacco Laboratory of Shandong Agricultural University (Tai'an, China) [1]. HR was examined after 24 h.

We also investigated the induction of resistance for tobacco mosaic virus. Harpin$_{Ea}$ and purified harpin$_{Xoo}$ were dissolved in distilled water to give a concentration of 30 μg/ml, and sprayed on nine leaves of three *N. tabacum* L. cv. Xanthi tobacco plants. Water was used as a control. Tobacco plants were grown in a greenhouse for 6–7 weeks before use. After harpin's being sprayed for 16 h, the tobacco mosaic viruses, kindly provided by Dr. Yi-Jun ZHOU (Jiangsu Academy of Agricultural Science, Nanjing, China), were inoculated according to the method described by Fang [25]. Three to four days later, the plants were assayed for typical necrotic lesion production.

harpin$_{Xoo}$ was visualized as multiple bands by SDS-PAGE, but visualized as a single band after a boiling water bath (**Fig. 2**, lanes 2 and 3). The yield was about 6 mg harpin$_{Xoo}$ per liter of culture.

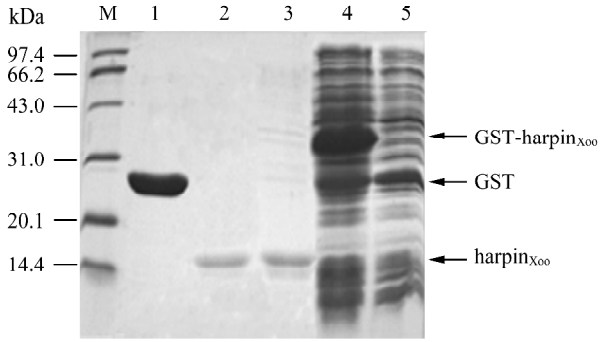

Fig. 2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of expression and purification of harpin$_{Xoo}$ protein in *Escherichia coli*

M, mid-range protein marker; 1, the purified glutathione S-transferase (GST) from *E. coli* BL21/pGEX-hpa1; 2, the purified harpin$_{Xoo}$ from *E. coli* BL21/pGEX-hpa1 (bathed with boiling water); 3, the purified harpin$_{Xoo}$ from *E. coli* BL21/pGEX-hpa1 (not boiled); 4, the supernatant of *E. coli* BL21/pGEX-hpa1; 5, the supernatant of *E. coli* BL21/pGEX-2T.

Results

GST-harpin$_{Xoo}$ fusion protein was expressed in the presence of IPTG and more soluble GST-harpin$_{Xoo}$ protein was obtained in culture conditions at 28 ºC than that at 37 ºC for an additional 4 h after 0.1 mM IPTG was added (**Fig. 1**). The crude GST-harpin$_{Xoo}$ protein was purified by the Bulk GST purification module, and the GST tag was removed by thrombin (**Fig. 2**). The normally purified

The polyclonal antibody against harpin$_{Xoo}$ was produced. The titer of the antibody against harpin$_{Xoo}$ reached 1:3000. Western blot analysis showed the antibody could be used to detect the purified harpin$_{Xoo}$, harpin$_{Xoo}$ in the total protein of *E. coli* BL21/pGEX-hpa1, or harpin$_{Xoo}$ from the *hpa1* transgenic rice line TR19 as reported in [26] (**Fig. 3**). A band with a molecular weight of 14 kDa was detected in

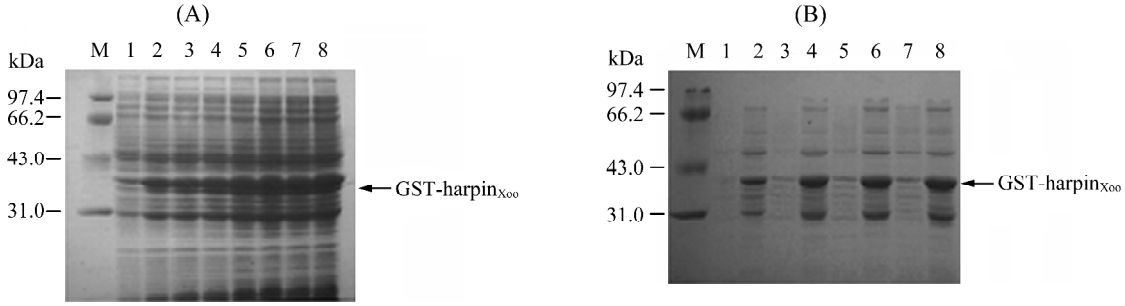

Fig. 1 Expression of the recombinant glutatione S-transferase (GST)-harpin$_{Xoo}$ fusion protein

(A) GST-harpin$_{Xoo}$ expression at different isopropyl-β-*D*-1-thiogalactopyranoside (IPTG) concentrations (induced at 28 ºC). M, mid-range protein marker; the IPTG concentrations of 1, 2, 3, 4, 5, 6, 7 and 8 were 0.01, 0.03, 0.05, 0.07, 0.1, 0.2, 0.5 and 1 mM, respectively. (B) GST-harpin$_{Xoo}$ expression at different temperatures and inducing times (0.1 mM IPTG). M, mid-range protein marker; 1, 3, 5 and 7 were GST-harpin$_{Xoo}$ protein induced at 37 ºC for 1, 2, 3 and 4 h, respectively; 2, 4, 6 and 8 were GST-harpin$_{Xoo}$ protein induced at 28 ºC for 1, 2, 3 and 4 h, respectively. Samples were the supernatants of sonicated suspensions.

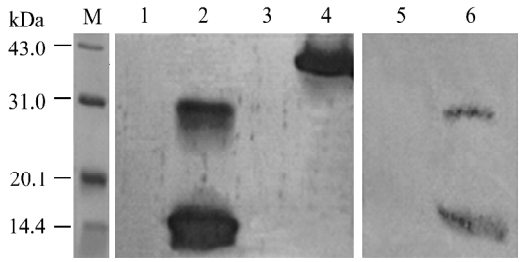

Fig. 3 Western blot analysis of harpin$_{Xoo}$ expression in *Escherichia coli* and transgenic rice

1, the purified glutathione S-transferase (GST) from *E. coli* BL21/pGEX-hpa1; 2, the purified harpin$_{Xoo}$ from *E. coli* BL21/pGEX-hpa1; 3, the total protein of *E. coli* BL21/pGEX-2T; 4, the total protein of *E. coli* BL21/pGEX-hpa1; 5, the total protein of rice variety R109; 6, the total protein of a transgenic line TR19 generated from rice variety R109; M, mid-range protein marker.

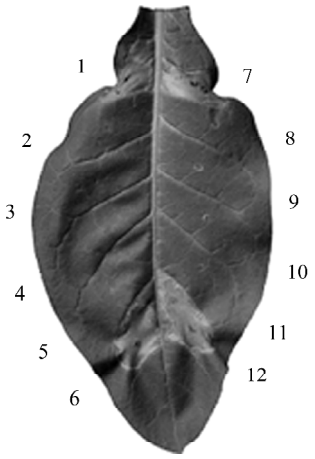

Fig. 4 Metabolism inhibitors and proteinase K inhibited the ability of harpins to induce a hypersensitive response in tobacco

1, harpin$_{Xoo}$ heat-treated at 100 ºC for 10 min; 2, harpin$_{Xoo}$ plus 1×10^{-4} M cycloheximide; 3, harpin$_{Xoo}$ plus 7.1×10^{-5} M actinomycin; 4, harpin$_{Xoo}$ plus 1×10^{-3} M lanthanum chloride; 5, harpin$_{Xoo}$ treated with protease K; 6, harpin$_{Xoo}$ treated with protease K plus phenyl methyl sulphonyl fluoride (PMSF); 7, harpin$_{Ea}$ heat-treated at 100 ºC for 10 min; 8, harpin$_{Ea}$ plus 1×10^{-4} M cycloheximide; 9, harpin$_{Ea}$ plus 7.1×10^{-5} M actinomycin; 10, harpin$_{Ea}$ plus 1×10^{-3} M lanthanum chloride; 11, harpin$_{Ea}$ treated with protease K; 12, harpin$_{Ea}$ treated with protease K plus PMSF. The concentration of harpin$_{Xoo}$ or harpin$_{Ea}$ is 10 μg/ml.

all materials presumed to contain harpin$_{Xoo}$. The molecular weight of 14 kDa was equal to the expected size based on sequence data of gene *hpa1*. A band of about 40 kDa was found in the total protein of *E. coli* BL21/pGEX-hpa1, which should be the GST-harpin$_{Xoo}$ fusion protein (the molecular weight of GST is about 26 kDa). An additional band was found with a molecular weight of 29 kDa, more than double that of the 14 kDa band. This band might be a dimer of harpin$_{Xoo}$. No bands appeared in negative controls, including the purified GST, the total protein of *E. coli* BL21/pGEX-2T and rice variety R109. These results indicated that the polyclonal antibody developed in this experiment was specific against harpin$_{Xoo}$.

The purified harpin$_{Xoo}$ lost the ability to elicit HR after treatment with protease K but could still elicit HR after heat treatment in a boiling water bath and treatment with protease K+PMSF, suggesting that harpin$_{Xoo}$ is sensitive to protease K but stable to heat treatment (**Fig. 4**). The capacity of harpin$_{Xoo}$ to induce HR was restrained after treatment by various plant metabolic inhibitors (**Fig. 4**). These results indicate that the HR induced by harpin$_{Xoo}$ requires active plant metabolism and is not a result of direct toxicity.

Both harpin$_{Ea}$ and harpin$_{Xoo}$ could induce tobacco mosaic virus (TMV) resistance in tobacco, but harpin$_{Xoo}$ was more effective than harpin$_{Ea}$ (**Table 1**).

Table 1 Induction for resistance to tobacco mosaic virus by harpin$_{Xoo}$ and harpin$_{Ea}$

Group	Lesion number	Control efficiency (%)
Harpin$_{Xoo}$	20.6±8.8*	78.8±0.1
Harpin$_{Ea}$	34.6±10.5*	64.4±0.1
Water	97.3±11.1	–

*P<0.05 vs. water group.

Discussion

Harpins are a special family of proteins produced by plant bacteria. They are able to elicit HR on some non-host plants and, at the same time, induce a series of defense reactions. Recently, evidence has shown that crude harpins from two pathovars of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Xooc) have these basic properties [20]. HR, in general, is different from damage caused by toxins. HR is a rapid cell apoptosis induced by avirulent pathogens or elicitors produced by avirulent pathogens [27].

In our previous study, the *hpa1* gene was inserted into pET-30a(+), and harpin$_{Xoo}$ was expressed in *E. coli*, but not purified, and antibody against harpin$_{Xoo}$ was not produced [11,20]. In this research, the GST gene fusion system was used and a large amount of purified harpin$_{Xoo}$ protein (up to 6 mg per liter of culture) was obtained. The inhibition of harpin$_{Xoo}$-induced HR by some plant metabolic inhibitors and the induction of TMV resistance, together with the physical characteristics of harpin$_{Xoo}$, suggest that

the protein we purified is a typical harpin, similar to harpin$_{Pss}$ [9] and harpin$_{Ea}$ [1]. The purification of harpin$_{Xoo}$ and production of its antibody make it possible to further study on its structure and function, as well as monitor the protein produced in recombinant microorganisms or transgenic plants.

Previous reports indicate that the plant-pathogenic bacteria do not produce harpins in culture because they are induced in nutrient-poor conditions that mimic the plant apoplast [28–32]. Recently, two harpin-like proteins have been isolated in a small amount from bacterial cells of Xoo and Xooc cultured in NB medium [33]. In addition, abundant pigment and lipopolysaccharide produced by Xoo make it difficult to purify the harpin$_{Xoo}$ from Xoo [34,35]. Thus, the purification of harpin$_{Xoo}$ from *E. coli* is easier than from Xoo.

Our result showed harpin$_{Xoo}$ was more effective than harpin$_{Ea}$ in inducing TMV resistance in tobacco. It has also been reported that harpin$_{Xoo}$ shows better results than harpin$_{Ea}$ in controlling *Fusarium* wilt and *Verticillium* wilt on cotton in the field [36]. The probable reason was that the molecular weight of harpin$_{Xoo}$ (14 kDa) is smaller than that of harpin$_{Ea}$ (44 kDa), and the molarity of harpin$_{Xoo}$ would be higher than that of harpin$_{Ea}$ at the same concentration (*W*/*V*) of protein solution.

We found that GST-harpin$_{Xoo}$ fusion proteins expressed in culture conditions at 28 ℃ were more soluble than those at 37 °C. The possible reason is that the lower temperature is favorable to the accurate folding of GST-harpin$_{Xoo}$ fusion proteins [37].

The hypothesized dimer of harpin$_{Xoo}$ was found in Western blot analysis of this study. A similar phenomenon was reported in harpin$_{Pss}$ when it was dissolved in a neutral buffer system [38]. The possibility of dimer formation of harpin$_{Xoo}$ may be due to incomplete protein denaturation. Wu *et al*. [39] reported that the NHR3 domain of the ETO protein was a tight tetramer, but four bands, representing tetramer, trimer, dimer and monomer, could be observed by SDS-PAGE, although the loaded sample was boiled for several minutes at 90 °C. They proposed that the oligomerization of the NHR3 domain might be due to the combining strength of elements such as salt bridge, hydrophobic strength and hydrogen bond, because the NHR3 domain contains no cysteines and does not involve formation of a disulphide bond [39]. However, a cysteine present at position 45 at the N terminus of harpin$_{Xoo}$ [20] may form disulfide bond, leading to the formation of harpin$_{Xoo}$ dimer. The formation of disulfide bond would be the main reason of dimer in transgenic rice. The oligomerization of harpin may affect the interaction between harpin and the plant cell membrane [38]. The similar function of the oligomerization of animal bacterial protein toxins such as α-toxin, streptolysin-O and hemolysin, has been reported [40].

In summary, we have expressed and purified a harpin protein in *E. coli* and produced polyclonal antibody against it. The purified protein has similar biological characteristics as the previously reported crude harpin$_{Xoo}$ [11] and harpin$_{Ea}$ [1], indicating that the protein we purified is harpin$_{Xoo}$. The polyclonal antibody has been successfully used in detection of harpin$_{Xoo}$ expression in transgenic rice.

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