A Highly-Conserved Single-Stranded DNA-Binding Protein in Xanthomonas Functions as a Harpin-Like Protein to Trigger Plant Immunity

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Abstract

Harpins are produced by Gram-negative phytopathogenic bacteria and typically elicit hypersensitive response (HR) in non-host plants. The characterization of harpins in Xanthomonas species is largely unexplored. Here we demonstrate that Xanthomonas produce a highly conserved single-stranded DNA-binding protein (SSBx) that elicits HR in tobacco as by harpin Hpa1. SSBx, like Hpa1, is an acidic, glycine-rich, heat-stable protein that lacks cysteine residues. SSBx-triggered HR in tobacco, as by Hpa1, is characterized by the oxidative burst, the expression of HR markers (HIN1, HSR203J), pathogenesis-related genes, and callose deposition. Both SSBx and Hpa1-induced HRs can be inhibited by general metabolism inhibitors actinomycin D, cycloheximide, and lanthanum chloride. Furthermore, those HRs activate the expression of BAK1 and BIK1 genes that are essential for induction of mitogen-activated protein kinase (MAPK) and salicylic acid pathways. Once applied to plants, SSBx induces resistance to the fungal pathogen Alternaria alternata and enhances plant growth. When ssbx was deleted in X. oryzae pv. oryzicola, the causal agent of bacterial leaf streak in rice, the resulting ssbx mutant was reduced in virulence and bacterial growth in planta, but retained its ability to trigger HR in tobacco. Interestingly, ssbx contains an imperfect Pip-box (plant-inducible promoter) and the expression of ssbx is regulated by HrpX, which belongs to the AraC family of transcriptional activators. Immunoblotting evidence showed that SSBx secretion requires a functional type-III secretion system as Hpa1 does. This is the first report demonstrating that Xanthomonas produce a highly-conserved SSBx that functions as a harpin-like protein for plant immunity.

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Introduction

Plants employ innate immune systems to overcome microbial pathogen infections [1,2]. Pathogen-associated molecular patterns (PAMPs) comprise a diverse group of molecules such as flagellin [3], EF-Tu [4], chitin [5] and harpins [6–8]. PAMPs are known to elicit plant-triggered immunity (PTI); briefly, PAMPs are recognized by plasma membrane-localized receptor-like kinases (RLKs), which often contain nucleotide-binding domains and leucine-rich repeats [9–11]. Examples of RLKs include flagellin-sensitive 2 (FLS2) [12], the EF-Tu receptor EFR [13], and the chitin elicitor receptor kinase 1 (CERK1) [14]. These RLKs take similar roles to proteins encoded by plant resistance (R) genes for pathogen recognition [9,10,15,16].

PTI is a form of basal defense or non-host-mediated resistance in plants. PTI and effector-triggered immunity (ETI) activate similar signaling pathways and defense responses in plants. However, ETI generally activates a more prolonged, robust resistance than PTI [2]. Signal transduction pathways associated with PTI and ETI include mitogen-activated protein kinase (MAPK) cascades, calcium fluxes, and the activation of reactive oxygen species (ROS). Furthermore, both ETI and PTI are associated with modulations in hormonal signaling pathways including those associated with production of salicylic acid (SA) for systemic acquired resistance (SAR), jasmonic acid (JA) for induced systemic resistance (ISR) and ethylene (Eh) [17]. Unlike ETI, PTI-modulated signaling requires BAK1, which is a BRI1-ASSOCIATED KINASE 1 that regulates plant signaling by functioning as an adaptor for multiple RLKs [2,17–20]. For example, the FLS2/BAK1 complex phosphorylates BIK1 (Botrytis-induced kinase 1) for signal transduction to the MAPK cascade [21]. The latter may then activate the expression of WRKY transcription factors that regulate SA-, JA- or Eh-dependent genes by binding the W-box [22]. However, it is unclear whether the proteins mentioned above are also involved in harpin-triggered immunity.

Recent studies have demonstrated that the α-helical structure of harpins is essential for HR induction, ion-mediated pore formation, development of curvilinear protofibrils or fibrils (amyloidogenesis), membrane-binding activities, ROS production and callose disposition [23–27]. Furthermore, multiple genes are
activated in harpin-treated tobacco including those involved in hormone signaling [28,29], HR markers (e.g. HIN1 and HSR203J) [30,31] and pathogenesis-related (PR1a and PR1b) [31–34]. Multiple reports document that harpin application promotes plant growth and induces SAR and ISR both to plant pathogens [28,34] and insects [29,35]. However, no reports have shown that harpin-elicited HR has any association with BAK1 in PTI-mediated signaling pathways. Although the elicitation of HR in resistant host plants is commonly associated with ESI, it also occurs during PTI [36]. Harpins, which are glycine-rich, heat-stable proteins produced by the type-III secretion system (T3SS), are PAMPs that elicit HR and PTI [27,37]. The first harpin described was HrpN, which is produced by the fire blight pathogen, Erwinia amylovora [7]. Multiple harpins can exist in a single phytopathogenic species; for example, Pseudomonas syringae pv. tomato DC3000 encodes four harpins, which are designated HrpZ1, HrpW1, HopA1K, and HopP1 [25]. In Ralstonia solanacearum, three harpins, PopA1 [38], HrpW [39], and PopW [40] have been identified. The HR elicited by harpins can be suppressed by eukaryotic metabolic inhibitors [6,41,42]. In Xanthomonas, the first harpin reported is HpaG in X. axonopodis pv. glycines [43], homologous to Hpa1 of X. oryzae pv. oryzae and X. oryzae pv. oryzicola, and to XopA of X. campestris pv. vesicatoria [6,43], but the latter does not elicit a HR in tobacco [43]. Interestingly, a hpa1 deletion mutant still triggers a HR on nonhost tobacco [6,44,43], indicating that uncharacterized HR-elicitors are present in X. oryzae pv. oryzicola.

The genus Xanthomonas contains 307 species or pathovars that infect at least 124 monocotyledonous and 268 dicotyledonous plants and causes enormous agricultural losses [46]. Despite the huge host range of Xanthomonas, few species in this genus are known to cause disease on tobacco, suggesting that tobacco may sense a conserved molecule in Xanthomonas and potentially initiate plant immunity. In this report, we present evidence that a highly-conserved single-stranded DNA-binding protein (SSBX) in Xanthomonas is regulated by HrpX, secreted via the T3SS, required for SAR and ISR both to plant pathogens [28,34]. These novel results indicate that SSBX functions as a harpin-like protein and modulates plant immunity in tobacco.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

The bacterial strains and plasmids used in this study are listed in Table S1. The wild-type strains X. oryzae pv. oryzae RS105, X. oryzae pv. oryzae PXO99A, X. campestris pv. vesicatoria 85–10, X. axonopodis pv. citri 306, X. campestris pv. campestris 8004, R. solanacearum ZJ3721 and E. amylovora 0065 (Table S1) were grown on nutrient agar (NA) or in nutrient broth (NB) [44] at 28°C. P. syringae DC3000 was grown on King’s Medium B [47]; E. coli and A. tumefaciens GV3101 were grown in Luria-Bertani (LB) medium [48] at 37°C and 28°C, respectively, hrp-inducing media included XOM3 for X. oryzae strains [49], XVM2 for X. campestris pv. vesicatoria 85–10 and X. axonopodis pv. citri 306 [50], and MMX for X. campestris pv. campestris 8004 [51]. MS medium was used for germination of plant seeds [52]. Antibiotics were used at the following concentrations (µg/ml): ampicillin (Ap), 100; kanamycin (Kn), 50; rifampicin (Rif), 50; and spectinomycin (Sp), 100 µg/ml.

**DNA manipulation.** DNA isolations, subcloning, transformation, PCR, Northern blot and immunoblotting were conducted using standard procedures [53]. PCR primers are described in Table S2. PCR products were first cloned into pMD18-T (TaKaRa, China) and then verified by sequencing. DNA sequences were analyzed with the VECTOR NTI software (http://www.invitrogen.com).

**Determination of SSBX-elicited HR in planta.** To investigate whether sbbx triggers HR in tobacco, full-length sbbx (552 bp) was amplified by PCR with the primer pairs sbbX-F/ssbX-R (Table S2) using the genomic DNA of strain RS105 as template. The amplified product was then cloned into PVX vector pgR107 [54] at Cidl and SfiI sites, resulting in pPVX-sbxhpa1 (Table S1). The hpa1 [55] and hpa2 [56] genes were also cloned into pgR107, generating pPVXhpa1 and pPVXhpa2 (Table S1), which were used as controls. These constructs (along with the empty vector) were transfected into A. tumefaciens GV3101, resulting in strains SSBx, Hpa1, Bax and PVX, respectively. Suspensions of A. tumefaciens strains were adjusted to OD600 = 0.5 and infiltrated into N. benthamiana with needleless syringes. HR symptoms were scored 48 hours post inoculation (hpi). Three independent experiments were performed and similar results were yielded. Representative results from one of these experiments are presented here.

**SSB Protein Expression and Purification**

SSBX, homologues were amplified from X. oryzae pv. oryzae RS105, X. oryzae pv. oryzae PXO99A, X. campestris pv. vesicatoria 85–10, X. axonopodis pv. citri 306, X. campestris pv. campestris 8004, R. solanacearum ZJ3721 and E. amylovora 0065, E. coli BL21(DE3), P. syringae pv. tomato DC3000, and P. fluorescens (Table S1). Each sbbx gene was amplified by PCR from corresponding genomic DNAs using the primers listed in Table S2. PCR products were then cloned into pET41a (+) resulting in constructs designated pSSBXa, pSSBXb, pSSBXC, pSSBXD, pSSBXE, pSSBXF, pSSBXG, pSSBXH, and pSSBXI respectively (Table S1). These constructs were transfected into E. coli BL21(DE3) (Table S1) as recommended in the Novagen pET System manual (Novagen, USA). Proteins were expressed as recommended by Novagen. Briefly, a single colony of each recombinant strain was inoculated to 2 ml LB broth containing Km. After incubation at 37°C for 12 h, 2 ml of culture was transferred into 200 ml of fresh LB liquid containing 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) and incubated for 4 h at 37°C. Cells were harvested by centrifugation, and pellets (1 g) were resuspended in 5 ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4, pH 7.4); the solution also contained 20% glycerol, 5 U/ml DNAseI and 5 µl PMSF (phenylmethylsulfonylfluoride). Bacterial cells were lysed by sonication (20 kHz, 10 min). After centrifugation, (15,000 x g) for 15 min at 4°C, the supernatants were purified using a GSTrapTM FF column as recommended by the manufacturer (Purification Manual, GE Healthcare, Germany). The purified proteins were digested by thrombin to remove the GST-tag, and the purified proteins were quantified with the Easy Protein Quantitative Kit (TransGen Biotech, China) and a NANOdrop 1000 Spectrophotometer (Thermo). Purified proteins were then used for HR induction assays in tobacco. The Hpa1 protein purified and the empty vector preparation (EVV) by the same procedure was used as a positive and negative control, respectively.

**HR assays.** Purified proteins were tested for their ability to elicit HR on N. benthamiana or N. tabacum cv. Xanthii by infiltration into plant tissues using needleless syringes. Plant responses were observed 48 hpi for the HR. All plants were grown in growth chambers at 25°C with a 12 h photoperiod. Experiments were repeated at least three times.

To measure minimal HR-eliciting concentrations, purified SSBX, and others were diluted in PBS buffer at the following concentrations: 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.1, 0.05 and 0.01 µM, while the purified Hpa1 diluted in PBS at the same
concentrations above was used as positive control. All concentrations of the tested SSB proteins and Hpa1 were infiltrated into tobacco leaves and photographed 48 hpi.

To characterize biochemical activity, purified SSBXoc (1 µM) and Hpa1 (1 µM) were heat-treated at 100°C for 10 min and incubated with protease K (0.5 U/ml) at 37°C for 10 min, respectively. To investigate potential susceptibility to eukaryotic metabolic inhibitors, SSBXoc (1 µM) and Hpa1 (1 µM) were mixed with 1 mM LaCl₃, 0.71 µM actinomycin D, and 0.1 µM cycloheximide, respectively. Treated and untreated SSBXoc, Hpa1, and EVP were infiltrated into tobacco leaves. Three independent biological experiments were performed and yielded similar results. Representative results from one of these experiments are presented.

To determine whether the HR induced by SSBXoc was dependent on SA accumulation in plants, purified SSBXoc (1.0 and 5.0 µM) was infiltrated into wild-type and NahG tobacco, respectively. Purified Hpa1 (1.0 and 5.0 µM), wild-type strain RS105 (OD₆₀₀ = 0.5), and EVP were used as controls. Three independent biological experiments were performed and yielded similar results. Representative results from one experiment are presented in this report.

**DNA laddering assays.** Genomic DNA of cv. Xanthi leaves infiltrated with purified SSBXoc (1 µM), Hpa1 (0.5 µM) and PBS buffer was isolated at 3, 6, 12, 24, 36 and 48 hpi, respectively. DNase-free RNase A was then used to digest existing RNA. Genomic DNA (10 µg) from each sample was subjected to electrophoresis in 2% agarose gels for at least 10 h under low voltage. Three independent experiments were performed and similar results were yielded. Representative results from one experiment are shown here.

**H₂O₂ assays.** Tobacco leaves (N. benthamiana) were infiltrated with purified SSBXoc (1 µM), Hpa1 (0.5 µM), and EVP, respectively, using needleless syringes. Eight hours later, treated leaves were collected and incubated in diaminobenzidine (DAB) for 8 h at 25°C, and then boiled in 95% ethanol for 10 min to remove the dye [56]. After 4 h further incubation in ethanol, leaves were fully bleached and brown precipitates were observed, indicating H₂O₂ accumulation and the production of ROS. Epidermal peels were performed at the injection sites 0 and 8 hpi with purified SSBXoc (1 µM), Hpa1 (0.5 µM), and EVP; these were then stained with DAB for 8 h at 25°C, and visualized with an Olympus IX71 microscope. Three independent experiments were performed and similar results were yielded. Representative results from one experiment are presented here.

**Callose deposition assays.** To observe callose deposition, tobacco leaves (N. benthamiana) were infiltrated with purified SSBXoc (1 µM), Hpa1 (0.5 µM) and EVP, respectively, using needleless syringes. After infiltration (0 and 8 h), leaf epidermal peels of the infiltrated area were removed and incubated in aniline blue (0.1% in 0.15% K₂HPO₄, pH 8.2) for 0.5 h. Fluorescence (400 nm excitation and 510 nm emission) and bright-field images were captured using a confocal microscope. Representative results from one experiment are presented here.

**Northern blot assays.** Total RNA of N. benthamiana leaves infiltrated with SSBXoc (1 µM), Hpa1 (0.5 µM) and EVP, was extracted 8 hpi. Prior to electrophoresis, DNA samples were treated with RNase-free DNaseI (TaKaRa, China) to remove potential traces of genomic DNA. RNA samples (30 µg) were then separated by electrophoresis in 1% agarose gels. Biotin-labeled DNA probes were prepared with the BrightStar Psoralen-Biotin Labeling kit (Ambion, USA) as recommended by the manufacturer. The primers for DNA probes are listed in Table S2. RNA was transferred to Hybond N+ membranes (Amersham Pharmacia Biotech, USA), hybridized with specific probes (Table S2) at 42°C using Northern Max (Ambion, USA), and detected using BrightStar BioDetect (Ambion) according to the manufacturer’s instructions.

**Assays for Plant Growth Promotion and Disease Prevention by SSBXoc**

To detect potential plant growth promoting activity of SSBXoc, seeds of cv. Xanthi and Arabidopsis thaliana (Col-0) were treated with SSBXoc (1 µM), Hpa1 (0.5 µM) and EVP, and sterile water (DDW) for 8 h at 4°C. Treated seeds were placed on MS agar medium and measured for root length and fresh weight two weeks after treatment.

The potential effect of SSBXoc in enhancing plant disease resistance was investigated on tobacco inoculated with A. alternata strain TBA28A (Table S1), the causal agent of brown spot disease. Ten plants of two-month-old tobacco were spray-inoculated with SSBXoc (1 µM in 0.5% Tween 20 solution) or Hpa1 (0.5 µM); plants were sprayed twice in three-day intervals. EVP was used as a negative control. Three days after the second spray, plants were inoculated with A. alternata TBA28A fresh disc. Infection was measured as the diameter of necrotic brown spots by statistical analysis.

**Construction of sssbXoc deletion mutants.** Experiments were designed to generate nonpolar deletion mutants of sssbX in X. oryzae pv. oryzae strains RS105 and RΔhpa1, a hpa1 deletion mutant [45]. The DNA sequences flanking sssbX were amplified from RS105 genomic DNA using primer pairs sssbX-F/sss-b-R and sssbX-F/sss-bII-R (Table S2), cloned into pMD18-T (Takara, China), and verified by sequencing. After digestion with BamHI/Xhol and Xhol/PsiI, the two fragments were cloned into the suicide vector pKMS1 [57] at BamHIand PstI sites, resulting in pKAΔssbX (Table S1). This construct was introduced into the wild-type RS105 and the hpa1 deletion mutant RΔhpa1, and the isolation of sssbX deletion mutants was performed as described previously [57]. The sssbX deletion mutant RAΔssbX and the double mutant RΔhpa1ΔssbX were verified by PCR using primers ssbX-F/sss-bII-R (Table S2) and by Southern blot analysis using ssbX as a probe.

**Bacterial pathogenicity and HR assays.** Pathogenicity assays were performed as described previously [8]. X. oryzae pv. oryzae derivatives were examined for their ability to cause disease symptoms in rice IR24 (Oryza sativa ssp. indica) or to trigger a HR in tobacco cv. Xanthi. Rice adult plants (two-months-old) were inoculated by leaf-needling and fully-expanded tobacco leaves were infiltrated by needleless syringes with bacterial suspensions (∼3×10⁸ cfu/ml). Lesion lengths in rice were scored 14 days post-inoculation (dpi) and the HR in tobacco 2 dpi. All plants were maintained in growth chambers at 25°C with a 12 h photoperiod. Experiments were repeated at least three times.

**Measurement of bacterial growth in rice.** Bacterial cell suspensions (3×10⁸ cfu/ml; OD₆₀₀=0.3) were infiltrated into recently expanded leaves of two-week-old rice IR24 with needleless syringes at three spots per leaf. Three 0.8 cm diameter leaf discs were harvested with a cork borer from each infiltrated area. The leaf discs were surface sterilized with 70% ethanol first and then boiled in 1 ml of distilled water, serial dilutions were plated in triplicate on NA with appropriate antibiotics. Plates were incubated at 28°C for 3–4 days until single colonies could be counted. Bacterial numbers (cfu/cm²) were calculated, and standard deviations were determined using colony counts from three replicate plates in each of three samples per time point per inoculum. Experiments were repeated at least three times.
Promoter activity assays and quantitative real-time PCR. To construct a transcriptional fusion between the sbbX promoter and glucuronidase (GUS), the promoter region (~1 to ~350 bp) upstream of sbbX was amplified from the genomic DNA of X. oryzae pv. oryzae RS105 with the primer pair psbA-F/psbB-R (Table S2). This PCR product was then fused with the promoter-less gusA gene, which was obtained with primers gusA-F/gusA-R (Table S2). The sbbX-gusA fusion was then cloned into pUFR034 [58] at the EcoRI site, resulting in pPIPGUS (Table S1). In another experiment, a mutation was introduced into the PIP-box of the sbbX promoter using primers mpsbA-F/mpsbB-R (Table S2) and fused with gusA, resulting in pPPIPGUS (Table S1).

For GUS activity assays, X. oryzae pv. oryzae RS105 strain and hrp mutants were cultured in XOM3 to OD600 = 0.5. Bacterial cells were diluted and disrupted in sonication buffer (20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 5 mM EDTA, and 1% Triton X-100). GUS activities were determined every 30 min over a 3-h time period by measuring absorbance at 410 nm with p-nitrophenyl-D-glucuronide as the substrate [59]. One unit (U) was defined as 1 nmol of 4-methylumbellif erone produced per min per bacterium.

For quantitative real-time PCR analysis (qRT-PCR), the bacteria were cultured as described for the GUS activity assay in this report or cultured in rice suspension cells as described by Li et al. [60] or cultured in rice suspension cells as described by Li et al. [60]. Bacterial cells were cultured as described for the GUS activity assay in this report or cultured in rice suspension cells as described by Li et al. [60]. Bacterial cells were cultured as described for the GUS activity assay in this report or cultured in rice suspension cells as described by Li et al. [60].

Results

sbbXoc Encodes a Single-stranded DNA-binding Protein Eliciting HR in Tobacco

Mutagenesis of hrgG or hrgX in X. oryzae pv. oryzae abolishes the elicitation of HR in tobacco and pathogenicity in rice [8]. Thus, we assumed that the expression of HR-eliciting genes, including hpa1, are also regulated by HrpG and HrpX [60]. Using cDNA microarrays of X. oryzae pv. oryzae strain RS105 and the hrgG & hrgX mutants (unpublished), we discovered that the expression of XOC_1514, which encodes a single-stranded DNA-binding protein (AEQ53953.1) [61], was positively regulated by HrpG and HrpX in pathogen-infected rice cells (Fig. 1). This protein, which was designated SSBXoc, is rich in glycine (20% of the total amino acids) but lacks cysteine residues (Table S3, Fig. 3).

To confirm this, we expressed sbbXoc in PVX vector pgR107, which is typically used to screen HR elicitors in tobacco [54]. SSBXoc triggered HR in N. benthamiana that was similar to Hpa1 [8] and Bax [55] (Fig. 2A), suggesting that SSBXoc functions as a harpin in X. oryzae pv. oryzae.

Previously, we reported that the minimum concentration of Hpa1 for HR induction is 0.1 μM [8]. To determine the concentration of SSBXoc required for HR induction, we over-expressed the protein in E. coli BL21 (DE3) (Table S1). Purified SSBXoc was infiltrated into tobacco at concentrations ranging from 0.01 to 50 μM. The minimum concentration of SSBXoc needed for HR induction in tobacco cv. Xanthi was 1.0 μM, approximately 10-fold higher than the minimum effective concentration of Hpa1 (Fig. 2B).

Figure 1. Expression of sbbXoc is induced in rice suspension cells. Real-time quantitative PCR analysis of sbbXoc transcript levels in X. oryzae pv. oryzae wild-type RS105 and mutants ΔhrgG and ΔhrgX. Strains were grown in NB or rice suspension cells, and designated as (−) and (+), respectively. The ratios (shown in units of log2) reflect sbbXoc transcript levels between different strains in two different growth conditions. 1. +RS105; 2. −ΔhrgG/−RS105; 3. −ΔhrgG/+RS105; 4. +ΔhrgX/+RS105; 5. +ΔhrgX/−RS105. Data represent the means ± standard deviations (SD) from three replicates. doi:10.1371/journal.pone.0056240.g001
Figure 2. A highly conserved single-stranded DNA-binding protein (SSB) triggers a HR in tobacco. (A) HR induction by the SSBXoc protein of X. oryzae pv. oryzicola. A. tumefaciens GV3101 (OD600 = 0.5) containing hpa1, ssbX, and box genes in the PVX vector pgR107 was inoculated into N. benthamiana tobacco leaves with a needleless syringe. Hpa1 and Box were used as positive controls, and A. tumefaciens containing the empty vector PVX was used as a negative control. (B) Concentration of SSBXoc required for HR induction in tobacco cv. Xanthi. Purified SSBXoc was diluted into N. benthamiana tobacco leaves with a needleless syringe. Hpa1, which functions as a harpin of X. oryzae pv. oryzicola, was used as a positive control and EVP as a negative control. (C) HR assays in tobacco inoculated with SSBX homologues obtained from various bacterial species. SSB proteins were overproduced in E. coli, purified (see Methods), and diluted in PBS buffer to different concentrations from 0.01 to 50 μM. A typical image of HRs on tobacco leaves caused by the proteins at 1 μM was taken in this report. Numbers represent sections of leaves inoculated with the following: 1, EVP; 2, SSBXoc from X. oryzae pv. oryzicola RS105; 3, SSBXoo from X. oryzae pv. oryzicola PXO99A; 4, SSBXcc from X. axonopodis pv. citri 306; 5, SSBXoo from X. campestris pv. vesicatoria 85-10; 6, SSBXoc from X. campestris pv. campestris 8004; 7, SSBXo from P. fluorescens Pf-5; 8, Hpa1Xoc from X. oryzae pv. oryzicola RS105; 9, SSBXo from E. amylovora 0065; 10, SSBXo from E. coli BL21 (DES); 11, SSBXo from R. solanacearum ZJ2731; and 12, SSBXo from P. syringae pv. tomato DC3000. (D) Assays for SSBX and Hpa1-induced HR in response to various metabolic inhibitors. Tobacco plants were infiltrated with SSBX (1 μM) or Hpa1 (0.5 μM), which was heat-treated or incubated (see methods) with one of the following: 1 μM LaCl3, 0.71 μM actinomycin D, 0.1 μM cycloheximide or protease K (0.5 U/ml). Leaf panels: 1, Hpa1; 2, heat-treated Hpa1; 3, protease K-treated Hpa1; 4, Hpa1 plus 1 μM LaCl3; 5, Hpa1 plus 0.71 μM actinomycin D; 6, Hpa1 plus 0.1 μM cycloheximide; 7, SSBXo; 8, heat-treated SSBXoc; 9, protease K-treated SSBXoc; 10, SSBXoc plus LaCl3; 11, SSBXoc plus 0.1 μM actinomycin D; 12, SSBXoc plus cycloheximide; 13, distilled water; and 14, EVP. Leaves in panels A to D were photographed 24–48 h after infiltration. (E) Analysis of DNA laddering in SSBXoc-treated tobacco leaves. Total genomic DNA was isolated from tobacco leaves 3, 6, 12, 24, 36 and 48 hpi with Hpa1 (0.5 μM) and EVP. DNA laddering was evaluated in 2% agarose gels. (F) Northern blot analysis in tobacco inoculated with SSBX, Hpa1, or EVP. The marker genes, HIN1, HSR203J, PR1a and PR1b, were chosen as the targets. Total RNAs were extracted from tobacco leaves infiltrated with SSBXoc (1 μM), Hpa1 (0.5 μM), or PBS buffer. Aliquots (10 μg each) of the extracted RNAs were separated in 1% agarose gels, transferred onto membranes, and analyzed by northern blotting. Blots were hybridized with digoxigenin-labeled HIN1, HSR203J, PR1a and PR1b cDNA. The experiment was conducted twice with similar results.

Nucleotide and protein searches using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that SSBXoc homologues exist in other bacteria. Protein sequence alignment of SSBXoc with homologues from other Gram-negative bacteria indicated that the differences of SSB proteins between Xanthomonas and other prokaryotic bacteria mainly existed in the glycine-rich regions (see rectangle marked with dashes, Fig. S1). A phylogenetic analysis showed that SSB proteins could be classified into one of three groups (Fig. S2). Group I contained SSB proteins from closely related Xanthomonas species, group II SSB homologues from Xylella fastidiosa, R. solanacearum, Thermus aquaticus, P. aeruginosa, and P. syringae pv. syringae, and group III from Candidatus Liberibacter asiaticus, P. fluorescens, E. amylovora, Dickeya dadantii, Escherichia coli and Shigella dysenteriae (Fig. S2). Percentages of glycine-rich amino acids of SSBX in X. oryzae pv. oryzicola RS105 strain and other bacteria are also shown in Table S3.

The bioinformatics analysis described above prompted us to investigate whether the SSB proteins from various bacterial species could elicit HR in tobacco. PCR was used to amplify ssb genes from X. oryzae pv. oryzicola PXO99A, X. campestris pv. campestris 8004, X. oryzae pv. oryzicola RS105, A. tumefaciens GV3101 (OD600 = 0.5) containing hpa1, ssb, and box genes in the PVX vector pgR107 was inoculated into N. benthamiana tobacco leaves with a needleless syringe. Hpa1 and Box were used as positive controls, and A. tumefaciens containing the empty vector PVX was used as a negative control. (B) Concentration of SSBXoc required for HR induction in tobacco cv. Xanthi. Purified SSBXoc was diluted into PBS buffer and inoculated into tobacco with needleless syringes. Hpa1, which functions as a harpin of X. oryzae pv. oryzicola, was used as a positive control and EVP as a negative control. (C) HR assays in tobacco inoculated with SSBX homologues obtained from various bacterial species. SSB proteins were overproduced in E. coli, purified (see Methods), and diluted in PBS buffer to different concentrations from 0.01 to 50 μM. A typical image of HRs on tobacco leaves caused by the proteins at 1 μM was taken in this report. Numbers represent sections of leaves inoculated with the following: 1, EVP; 2, SSBXoc from X. oryzae pv. oryzicola RS105; 3, SSBXoo from X. oryzae pv. oryzicola PXO99A; 4, SSBXcc from X. axonopodis pv. citri 306; 5, SSBXoo from X. campestris pv. vesicatoria 85-10; 6, SSBXoc from X. campestris pv. campestris 8004; 7, SSBXo from P. fluorescens Pf-5; 8, Hpa1Xoc from X. oryzae pv. oryzicola RS105; 9, SSBXo from E. amylovora 0065; 10, SSBXo from E. coli BL21 (DES); 11, SSBXo from R. solanacearum ZJ2731; and 12, SSBXo from P. syringae pv. tomato DC3000. (D) Assays for SSBX and Hpa1-induced HR in response to various metabolic inhibitors. Tobacco plants were infiltrated with SSBX (1 μM) or Hpa1 (0.5 μM), which was heat-treated or incubated (see methods) with one of the following: 1 μM LaCl3, 0.71 μM actinomycin D, 0.1 μM cycloheximide or protease K (0.5 U/ml). Leaf panels: 1, Hpa1; 2, heat-treated Hpa1; 3, protease K-treated Hpa1; 4, Hpa1 plus 1 μM LaCl3; 5, Hpa1 plus 0.71 μM actinomycin D; 6, Hpa1 plus 0.1 μM cycloheximide; 7, SSBXo; 8, heat-treated SSBXoc; 9, protease K-treated SSBXoc; 10, SSBXoc plus LaCl3; 11, SSBXoc plus 0.1 μM actinomycin D; 12, SSBXoc plus cycloheximide; 13, distilled water; and 14, EVP. Leaves in panels A to D were photographed 24–48 h after infiltration. (E) Analysis of DNA laddering in SSBXoc-treated tobacco leaves. Total genomic DNA was isolated from tobacco leaves 3, 6, 12, 24, 36 and 48 hpi with Hpa1 (0.5 μM), SSBXoc (1 μM) and EVP. DNA laddering was evaluated in 2% agarose gels. (F) Northern blot analysis in tobacco inoculated with SSBX, Hpa1, or EVP. The marker genes, HIN1, HSR203J, PR1a and PR1b, were chosen as the targets. Total RNAs were extracted from tobacco leaves infiltrated with SSBXoc (1 μM), Hpa1 (0.5 μM), or PBS buffer. Aliquots (10 μg each) of the extracted RNAs were separated in 1% agarose gels, transferred onto membranes, and analyzed by northern blotting. Blots were hybridized with digoxigenin-labeled HIN1, HSR203J, PR1a and PR1b cDNA. The experiment was conducted twice with similar results.

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X. axonopodis pv. citri (Xac) 306, X. campestris pv. vesicatoria 85-10, R. solanacearum ZJ3271, P. syringa pv. tomato DC3000, P. fluorescens Pf-5, E. amylovora 0065, and E. coli BL21 (DE3) (Table S1). sbi genes were amplified using the primers listed in Table S2, and then cloned into pET30a, generating pSSB constructs (Table S1) harbored by E. coli BL21 (DE3). The overproduced and purified SSB proteins at concentrations from 0.01 to 50 μM were infiltrated into tobacco cv. Xanthi with needleless syringes. Only did the SSB proteins from Xanthomonas elicited HR in tobacco, whereas those from other bacterial species did not (Fig. 2C), suggesting that only SSBX homologues, which are closely related and highly conserved in Xanthomonas (Fig. S1; Table S3), function as harpin.

Electrophoretic mobility shift assays (EMSA) demonstrated that SSBXoc from X. oryzae pv. oryzae, as the representative of these proteins in Xanthomonas bound randomly synthesized single-stranded DNAs (DNA1 and DNA2, Fig. S3). This is consistent with that the single-stranded DNA-binding protein is for ssDNA protection from nucleolytic digestion in bacterial cell viability [62], implying that SSBX of Xanthomonas rather than other plant pathogenic bacteria is coevolved to be recognized as a potential HR-elicitor by plants.

Previous reports indicate that harpin proteins are heat-stable and protease-sensitive [6,7,34,41,42]. To investigate these characteristic for SSBX, purified protein (1 μM) was incubated in a water bath at 100°C for 10 min and with protease K (0.5 U/ml) at 37°C for 10 min, while Hpa1 was used as positive control. Heat- or protease-treated SSBX was then inoculated into tobacco cv. Xanthi. At 4 hr, heat-treated SSBX still triggered HR in tobacco (Fig. 2D panel b), but protease-treated SSBX did not (Fig. 2D, panel 9).

SSBXoc-elicited HR is a form of Programmed Cell Death

The next experiments were designed to determine whether SSBXoc, like Hpa1, is toxic to plant cells or not and SSBXoc leads to a metabolic response that triggers HR. Three metabolic inhibitors were used: actinomycin D (inhibits eukaryotic RNA polymerase II), cycloheximide (targets 80S ribosomes), and LaCl3 (a calcium channel blocker). These inhibitors were incubated with purified SSBXoc (see Methods) and then assayed for HR induction in tobacco. All three inhibitors prevented the SSBXoc-elicited HR in tobacco when co-infiltrated with the purified SSBXoc (see Methods) and then assayed for HR induction in tobacco. All three inhibitors prevented the SSBXoc-elicited HR in tobacco when co-infiltrated with the purified SSBXoc (Fig. 2D, panels 12-14). These results indicated that the SSBXoc-elicited HR is an active process and requires de novo gene expression, protein synthesis and calcium flux across membranes. Thus, SSBXoc acts as an elicitor, like Hpa1, of HR but is not directly toxic to plant cells.

It is well-documented that harpin-elicited HR is a form of programmed cell death (PCD), which is accompanied by DNA laddering [63]. To determine whether the SSBXoc-elicited HR is a form of PCD, DNA laddering experiments were performed. Total genomic DNA from SSBXoc-infiltrated tobacco leaves were extracted at different time points after infiltration and analyzed on 2% agarose gels. As shown in Fig. 2E, DNA ladders were clearly observed in SSBXoc-inoculated leaves at 24 hpi, 12 h later than that in Hpa1-inoculated leaves. Thus, SSBXoc, like Hpa1 (Fig. 2E), elicits PCD that is characterized by DNA laddering.

We then investigated whether SSBXoc-elicited HR occurs with the activation of known HR marker genes including HIN1 (harpin-induced 1) [64], HSR203J [65], and the SA-dependent marker, PR1a [66]; the JA-dependent gene, PR1b [67], was also conducted. The expression of these genes was evaluated in tobacco leaves infiltrated with SSBXoc, Hpa1, and EVP 6 hpi. All four genes were induced in response to SSBXoc; however, Hpa1 did not induce the expression of PR1b (Fig. 2F). Transcripts started to accumulate 6 hpi with SSBXoc and Hpa1 and were expressed at high levels up to 24 h (data not shown). These findings indicate that SSBXoc-elicited HR was accompanied by the expression of HR markers and plant defense genes.

SSBXoc-elicited HR is Dependent on SA Accumulation

It has been reported that HR induction by harpins requires SA accumulation [28,68]. To investigate whether this is valid for SSBXoc-elicited HR, we utilized transgenic tobacco expressing NahG; this line produces salicylic hydroxylase which degrades SA and blocks its accumulation [69]. Purified SSBX and Hpa1 produced a HR in wild-type tobacco (Fig. 3B), but not in the NahG line (Fig. 3A). Thus, SSBXoc-induced HR relies on SA accumulation in planta, which is the case for other harpins. It is noteworthy that the wild-type RS105 of X. oryzae pv. oryzae still elicited HR in SA-deficient tobacco (Fig. 3A). Thus, in addition to SSBXoc and Hpa1, other unidentified HR-elicitor(s) exist(s) in X. oryzae pv. oryzae to trigger HR on tobacco in SA-independence.

SSBXoc Activates Plant Basal Defense

The oxidative burst, which involves the generation of ROS in response to microbial elicitors, occurs quite quickly in resistant plant cells [70,71]. Thus we investigated whether the oxidative burst is generated in SSBXoc-treated tobacco cells. At 8 hpi, DAB staining resulted in necrotic brown spots indicative of H2O2 production in both SSBXoc- and Hpa1-infiltrated leaves (Fig. 3C). Along with the oxidative burst, plants often mobilize multiple forms of basal defense to inhibit pathogen ingress, including cell wall deposition in cell walls [72]. To determine whether SSBXoc elicits callose deposition in tobacco, epidermal peels from SSBXoc-infiltrated tissue were stained with aniline blue and examined by fluorescence microscopy. Both SSBXoc- and Hpa1-infiltrated leaves showed evidence of callose deposition beginning at 8 hpi (Fig. 3D). Thus, SSBXoc, like Hpa1, functions as an elicitor of basal defense responses and stimulates callose deposition.

The oxidative burst and callose deposition in tobacco infiltrated with SSBXoc prompted us to speculate that SSBXoc may function as a PAMP and activate the expression of genes involved in PTI signaling pathways. Our results indicate that BAK1, BIK1 and MAP3K (Fig. 3E), and NPR1, EIN2, COI1 and PR4 (Fig. 3F) genes are activated in response to SSBXoc or Hpa1. The data show that SSBXoc triggers a cascade of events similar to those triggered by Fli22 [12,71,73,74], which leads to the oxidative burst and callose deposition and activates the expression of PR genes. These results support our presumption that SSBXoc acts as a PAMP like Hpa1.

SSBXoc Induces Plant Disease Resistance and Promotes Plant Growth

Tobacco infiltrated with SSBXoc shows elevated expression of SA- and JA-dependent genes, along with the oxidative burst and callose deposition (Fig. 2F; Fig. 3C, D). Therefore, we hypothesized that SSBXoc may stimulate induced resistance to pathogen infection. For this, we inoculated a fungal pathogen, A. alternata TBA28A (Table S1), causal agent of tobacco brown spot disease, to fully-expanded tobacco leaves that were previously spray-inoculated twice with SSBXoc (1 μM) in three-day intervals. The necrotic areas in tobacco leaves treated with SSBXoc were significantly smaller (P = 0.01, t test) than those observed on leaves inoculated with EVP (Fig. 4).

Like SBS71, we found that Hpa1 (0.5 μM) also induced similar resistance to A. alternata. The data suggest that both SSBX and Hpa1 induce SAR against pathogen infection.

Xanthomonas SSB Protein Acts as Harpins

The oxidative burst and callose deposition in tobacco infiltrated with SSBXoc prompted us to speculate that SSBXoc may function as a PAMP and activate the expression of genes involved in PTI signaling pathways. Our results indicate that BAK1, BIK1 and MAP3K (Fig. 3E), and NPR1, EIN2, COI1 and PR4 (Fig. 3F) genes are activated in response to SSBXoc or Hpa1. The data show that SSBXoc triggers a cascade of events similar to those triggered by Fli22 [12,71,73,74], which leads to the oxidative burst and callose deposition and activates the expression of PR genes. These results support our presumption that SSBXoc acts as a PAMP like Hpa1.
Figure 3. SSBXoc from X. oryzae pv. oryzicola may function as a PAMP and activates PTI in tobacco. SSBXoc-triggered HR depends on the accumulation of salicylic acid (SA). X. oryzae pv. oryzicola strain RS105 (OD600 = 0.5), SSBXoc (1.0 and 5.0 μM), Hpa1 (1.0 and 5.0 μM), and EVP were inoculated into a NahG tobacco leaves (A) or wild-type tobacco cv. Xanthi (B). Photographs were taken 48 hpi. (C) SSBXoc-triggered HR is accompanied by the oxidative burst. The production of H2O2 was evaluated in tobacco leaves by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The reaction mixture contained 200 μl of 0.5 mM DAB in 50 mM Tris acetate buffer (pH 6.0) with purified SSBXoc (1 μM) or Hpa1 (0.5 μM). Fully-expanded tobacco leaves were infiltrated with needleless syringe, and PBS buffer was used as a negative control. RNA was extracted 8 hpi and 10

Figure 4. SSBXoc induces resistance to tobacco brown spot disease caused by A. alternata. Fully-expanded tobacco leaves (cv. Xanthi) were sprayed twice in three-day intervals with purified SSBXoc (1 μM), Hpa1 (0.5 μM) and EVP (negative control). Three days after the second spraying (dpi), SSBXoc leaves were inoculated with A. alternata strain TBA28A. Diameter of brown spot lesions were measured and photographed 14 dpi. Lesion size (diameter) are shown ± SD of triplicate measurements. Different letters above columns indicate significant differences at P=0.01 using the Student’s t test. doi:10.1371/journal.pone.0056240.g004

ssbXoc is Required for Full Virulence and Bacterial Growth in Rice

To investigate the potential contribution of SSBX to virulence, ssbXoc was deleted both in X. oryzae pv. oryzicola RS105 and the hpa1 deletion mutant, RAhpa1 (Table S1). The RAΔssbXΔhpa1 double mutant (Table S1) was constructed using a two-step integration procedure [57]. Inoculation studies were conducted by inoculating one half of a rice leaf with wild-type RS105 and the remaining half with one of the following: ssbXoc deletion mutant RAΔssbX, hpa1 mutant RAΔhpa1 [44], the double mutant RAΔssbXΔhpa1 (Table S1), the complemented strain CRASBX and the T3SS mutant RAhreV [76]. Symptoms in RAΔssbX-inoculated leaves were reduced relative to the wild-type strain, but were not as attenuated as RAΔhpa1-mediated symptoms (Fig. 6A). Lesion lengths in RAΔssbX-inoculated leaves were significantly smaller than those induced by the wild-type RS105 (P=0.01, t test) but larger than those induced by RAΔhpa1 (Fig. 6B). The double mutant RAΔssbXΔhpa1 did not lose pathogenicity in rice (Fig. 6A), but lesions were significantly smaller than those induced by the wild-type and single mutants (RAΔssbX and RAΔhpa1) (Fig. 6B). As expected, the T3SS mutant, RAhreV, produced no obvious disease symptoms in rice (Fig. 6A, B). Disease lesion lengths for the complemented strain CRAssbX were equivalent to those induced by the wild-type RS105 (Fig. 6A, B), indicating that the mutant could be complemented for symptoms with the ssbXoc gene in trans.

To determine whether ssbXoc contributes to growth of X. oryzae pv. oryzicola in rice, we compared the population dynamics of the wild-type RS105, RAΔssbX, CRAssbX, RAΔhpa1, RAΔssbXΔhpa1, and RAhreV. The populations of RAΔssbX were significantly lower than the wild-type RS105 beginning 2 dpi, but higher than the population of RAΔhpa1 and RAΔssbXΔhpa1 (Fig. 6C). Growth of RAΔssbX was restored to wild-type levels when ssbXoc was present in trans (Fig. 6C). These results indicated that ssbXoc, like hpa1, contributes to bacterial growth in planta, although the effect was not as pronounced as seen with the T3SS mutant (RAhreV) or RAΔhpa1. Furthermore, mutations in ssbXoc and hpa1 did not abolish the ability of the pathogen to elicit HR in tobacco (data not shown), implying that other HR elicitor(s) exist in X. oryzae pv. oryzicola.

SBX X Secretion is Dependent on a Functional TTSS

The T3SS deficient mutant RAhreV did not trigger HR in tobacco implies that HR elicitors, including SSBXoc and Hpa1, may be secreted via the T3SS. Bioinformatics analysis of SSBXoc did not show obvious T3SS secretion signals that are commonly found in T3SS effector proteins [77,78]; thus it was not clear whether SSBXoc secretion required a functional T3SS. We used immunoblotting and SSBXoc tagged with a c-Myc epitope to...
explore whether SSB\textsubscript{xoc} secretion was T3SS-dependent. The construct for expressing c-Myc-tagged SSB\textsubscript{xoc} was transferred into the wild-type and mutants defective in \textit{hrcV} (encodes an inner membrane component of the T3SS), \textit{hrcC} (encodes an outer membrane component), \textit{hrpE} (encodes protein subunits of the Hrp pilus), \textit{hpaB} and \textit{hpaP} (encode exit control proteins for T3SE secretion) \cite{8}. These mutants were designated \textit{RDhrcV}, \textit{RDhrcC}, \textit{RDhrpE}, \textit{RDhpaB} and \textit{RDhpaP} (Table S1), respectively. \textit{RDssbX} with the empty vector pUFR034 was used as a negative control.

When the wild-type RS105 and mutants \textit{RDhrcC}, \textit{RDhrcV}, \textit{RDhrpE}, were incubated in a \textit{hrp}-inducing medium XOM3 \cite{50} and examined by immunoblotting, the SSB\textsubscript{xoc}-c-Myc protein, like Hpa1-c-Myc protein (as a positive control), was found in the supernatants (SN) of the wild-type and \textit{RDhrpE}, but absent from the SN fraction of \textit{RDhrcV} and \textit{RDhpaP} (Fig. 6D, E). These results indicate that a functional T3SS is needed for secretion of SSB\textsubscript{xoc} and Hpa1. The SSB\textsubscript{xoc}-c-Myc protein was detected in the SNs and total extracts (TE) of \textit{RDhpaB} and \textit{RAhpaP}, indicating that HpaB and HpaP are not required for secretion of SSB\textsubscript{xoc} and Hpa1 (Fig. 6D, E). Moreover, the secretion of Hpa1 was not impaired by the mutagenesis in \textit{ssbXoc}, and vice versa (Fig. 6D, E).

***ssbX is positively regulated by HrpX.*** The down-regulated expression of \textit{ssbXoc} in the \textit{RΔhrpX} and \textit{RΔhrpG} mutants indicates that \textit{ssbXoc} is positively regulated by HrpX and HrpG (Fig. 1). To investigate this further, we used promoter prediction software (HUhttp://www.fruitfly.org/seq_tools/promoter.html) to analyze the \textit{ssbXoc} promoter region in the \textit{X. oryzae pv. oryzae} BLS256 genome \cite{61}. This analysis revealed an imperfect PIP-box (TTCGC-N19-TTCGT) upstream of the \textit{ssbXoc} start codon (Fig. 7A), suggesting that \textit{ssbXoc} may be regulated by HrpX \cite{79}. To determine whether \textit{ssbXoc} expression depends on the putative PIP-box and HrpX, we constructed a recombinant plasmid pPIPAGUS, which contains the \textit{ssbXoc} promoter region fused to a promoter-less gusA in pUFR034, resulting in pPIPAGUS (Fig. 7A, Table S1). A mutated \textit{ssbXoc} promoter (first two TT nucleotides replaced with AA, see Fig. 7A) was also fused to gusA in pUFR034, generating pPIPBGUS (Fig. 7A, Table S1). Plasmids pPIPAGUS and pPIPBGUS were transformed into the wild-type RS105 and mutants \textit{RAhrpX} and \textit{RAhrpG}, incubated in the \textit{hrp}-inducing medium XOM3, and GUS activities were measured. GUS activity of pPIPAGUS was significantly lower in the \textit{hrpG} and \textit{hrpX} mutants than in the wild-type strain (P=0.01, t test). GUS activity of the mutated \textit{ssbXoc} transcriptional fusion (pPIPBGUS) was similar in the wild-type, \textit{RΔhrpG}, and \textit{RΔhrpX} strains.

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\textbf{Figure 5. SSB\textsubscript{xoc} enhances plant growth.} (A) Phenotype of tobacco (cv. Xanthi) and \textit{Arabidopsis thaliana} (Col-0) grown on MS medium, 14 days after seed treatment with Hpa1 (0.5 \textmu M), SSB\textsubscript{xoc} (1 \textmu M), EVP, or double distilled water (DDW). Upper panel, tobacco; lower panel, \textit{Arabidopsis}. (B) Fresh weight and root length of treated plants. Upper panel, fresh weight; lower panel, root length. Data are means \pm SD of 50 randomly selected plants. Different letters above columns represent significant differences between treatments (P=0.01 by t test). doi:10.1371/journal.pone.0056240.g005
We also used real-time PCR to evaluate expression levels of ssbXoc in strains RS105, RΔhpa1, the double mutant RΔssbXΔhpa1, the complemented mutant CRAssbX, and the T3SS mutant RAhrhCV. Ten leaves were inoculated with each strain (OD600 = 0.3; approximately 3 × 10^8 cfu/ml) by leaf-needling, and the assay was conducted in triplicate. Bacterial leaf streak symptoms were photographed 14 dpi, and representative symptoms are shown (A). The average lesion lengths formed by the wild-type and mutants were measured 14 dpi (B), and data represent means ± SD from three replicates. Different letters in each data column indicate significant differences at P = 0.01 (t test). (C) Bacterial growth assays in planta. Strains (OD600 = 0.3) were infiltrated into leaves of rice seedlings (IR24, two-weeks old) with blunt-end plastic syringes, and the cfu/cm^2 of tissue was evaluated as described in Methods. Data represent means ± SD from three replications. (D) and (E) demonstrated the secretion of SSBXoc (D) and Hpa1 (E) are dependent on a functional T3SS of X. oryzae pv. oryzicola. This experiment utilized X. oryzae pv. oryzicola RS105 and strains containing mutations in the following genes: hrcV (RΔhrcV), hrcC (RΔhrcC), hreE (RΔhreE), hpaB (RΔhpaB), hpaP (RΔhpaP), hpa1 (RΔhpa1) and ssbXoc (RΔssbX) to express ssbXoc-c-myc or hpa1-c-myc fusion (see Methods). After incubation (8 h) in hrp-inducing medium XOM3, total cell extracts (TEs) and culture supernatants (SNs) were analyzed by SDS-PAGE and immunoblotted with an anti-c-Myc antibody. The immunoblotting assay was conducted twice, and similar results were obtained each time. For the detection of SSBXoc, the strain RΔssbX with the empty vector pUFR034 was used as a negative control (D).

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Our results suggest that the HR-eliciting SSBX protein is highly conserved in Xanthomonas species (Fig. S1, Fig. 2C), leading us to investigate whether PIP-box promoters drive ssbX expression in other xanthomonads or not. Interestingly, PIP-box promoters were identified upstream of the ssbX coding sequences in X. oryzae pv. oryzicola PXO99A, X. campesiris pv. vesicatoria 85-10, X. axonopodis pv. citri 306, and X. campesiris pv. campesiris 8004 (data not shown), implying that ssbX expression in these bacteria is also induced in planta. Thus, we performed real-time PCR to investigate the expression levels of ssbX in various Xanthomonas species grown in the hrp-inducing media (see Methods). ssbX expression was significantly
higher in hrp-inducing media than in the nutrient-rich media, which suggests that these genes have a functional PIP-box and are regulated by HrpX.

Discussion

In this report, we demonstrate that single-stranded DNA-binding proteins from Xanthomonas elicit HR in tobacco. This activity was not demonstrated with SSR proteins obtained from other prokaryotes, so it may be a unique feature of Xanthomonas. Like Hpa1, SSRXoc contributes both to bacterial growth and virulence in rice (Fig. 6) and also triggers programmed cell death (Fig. 2D). This is the first report that SSBX binds nonspecifically to single-stranded DNA from nucleases [62]. Unlike some SSBs with a very narrow distribution, such as Axl21 in X. oryzae pv. oryzae [80], SSRX may be widely distributed in Xanthomonas species. It has been proposed that PAMPs are conserved throughout classes or genera of microbes and contribute to general microbial fitness [81].

Harpins are generally highly constrained structures that are difficult for plant pathogenic bacteria to alter because they have evolved to help bacteria avoid recognition in plants. The first identified harpin, HrpN from E. amylovora [7], has been identified in related pathogens including Pantoea stewartii subsp. stewartii [82] and D. dadantii [83]. Another harpin, HrpZ, first identified in P. syringae pathovars [6,84], but was later shown to be present in nonpathogenic pseudomonads including P. putida and P. fluorescens [85]. The harpin HrpW, which contains harpin and pectate-lyase domains, is widely conserved across genera and has been identified in E. amylovora [41], P. syringae [42], D. dadantii [86], R. solanacearum [39], and X. campestris pv. campestris [46]. Interestingly, there is no HrpW homologue in the genomes of X. oryzae pv. oryzae, X. oryzae pv. oryzae, X. campestris pv. vesicatoria, or X. axonopodis pv. citri [46,61,87]. Thus, some harpins may have a more narrow distribution. For example, R. solanacearum contains an SSR protein but this does not elicit HR in tobacco (Fig. 2D), possibly because SSRX lacks the conserved glycine-rich region of SSBRX (Fig. S1).
and the pathogen causes bacterial wilt in tobacco. It is also interesting to recall that flag15, a truncated version of flagellar-derived flag22, does not act as an elicitor in Arabidopsis or Nicotiana benthamiana, while it is fully active in tomato [88]. In Xanthomonas, only Hpa1 [8,43] and SSBX (Fig. 2; this study) have been identified as harpins that elicit HRs in tobacco. However, the hpa1-ssbx double mutant RΔhpa1Δssbx still elicited HR in the wild-type and SA-deficient (nahG) tobacco plants (data not all shown), implying that Xanthomonas produces other elicitors that trigger a SA-independent HR. This double mutant is a valuable resource for identifying other harpin(s) that exist in tobacco, presumably because harpin proteins are secreted via the T3SS independently of HpaB and HpaP (Fig. 6D, 3F), which stimulate plant defense. SSBX-induced HR, like Hpa1-induced, could be blocked by eukaryotic metabolic inhibitors (Fig. 2D). It will be interesting to determine whether SSB proteins from diverse genera can elicit HR, and such studies will help us understand how pathogens recruit molecules that are instrumental for bacterial fitness and re-deploy them as agents for plant defense.

Although plant-associated microbes can potentially produce many molecules with conserved signatures, only a few PAMPs have been identified, and most of these trigger a similar set of responses. In the current study, we evaluated the expression of PTI signature genes, e.g. BAK1, BIK1, and MAPK [19,73,94]. The activation of these genes by Hpa1 and SSBX (Fig. 3) further supports the contention that PTI is a variant of ETI [18,81]. Recently, Xa21 of X. oryzae pv. oryzae, which is perceived by Xa21 in rice [80], was shown to be recognized by FLS2 in Arabidopsis [95]. Thus it remains possible that Hpa1 and SSBX, like Xa21, may recruit FLS2 in a receptor complex together with other receptors and adaptors that modulate PTI. The identity of receptors for Hpa1 and SSBX, and whether these PAMPs interact with FLS2 remains unclear.

Figure 8. Working model of SSBX function. SSBX, like Hpa1, which are regulated at the transcriptional level by HrpG and HrpX, may be secreted through the Hrp pilus (encoded by hrpE), but not translocated through the T3SS as other T3Ss (different white shapes), and then are possibly recognized by an unidentified receptor (question mark) which associates with BAK1. This interaction may result in phosphorylation of BIK1 and subsequent phosphotransfer to the MAPK cascade to activate the expression of genes involved in SA-, JA- and Eth-signaling pathways that lead to induced resistance (SA and/or ISR) accompanied by callose deposition on cell walls and enhanced plant growth. MAPK signaling regulates NADPH oxidase-dependent oxidative burst in the early stages of plant defense.

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Plant immune responses triggered by harpins are often associated with HR and SAR [6,7,24,28]. In the present study, we show that SSBX induces PCD (Fig. 2E), the oxidative burst (Fig. 3C), the expression of HR and SAR marker genes (Fig. 2F and 3F), and callose deposition (Fig. 3D), which stimulate plant defense. SSBX-induced HR, like Hpa1-induced, could be blocked by eukaryotic metabolic inhibitors (Fig. 2D). It will be interesting to determine whether SSB proteins from diverse genera can elicit HR, and such studies will help us understand how pathogens recruit molecules that are instrumental for bacterial fitness and re-deploy them as agents for plant defense.

There is abundant evidence in the literature showing that harpins display pleiotropic effects both on HR & SAR and also impact plant growth [28,29,96]. Our results also showed that Hpa1 and SSBX enhanced growth of Arabidopsis and tobacco (Fig. 5); this was correlated with increased expression of EIN2 and PR1 genes (Fig. 3F) that are essential for Eth-signaling [75]. This is consistent with the contention that Eth-signaling regulates the accumulation of the FLS2 receptor and is required for the oxidative burst leading to PTI [97]; thus, Hpa1- and SSBX-mediated plant immunity may also require Eth-signaling. Eth- and SA-signaling may be regulated by WRKY transcription factors that are phosphorylated by the MAPK cascade [98,99]. Nevertheless, SSBX may possibly have pleiotropic effects in plants.
totally understood, SSB<sub>x</sub> secretion depends on the functional T3SS, but does not depend on the presence of HpaB and HpaP. We hypothesize that, besides protecting ssDNAs from nucleases in bacterial cells, SSB<sub>x</sub> possibly like Hpa1, is secreted through the T3SS, but not translated into plant cells, and perceived in plant apoplast where it is recognized by an unknown receptor, possibly a plasma membrane-localized PAMP receptor-like kinase (RLKs) that recruits other proteins, like BAK1, and activates downstream signal transduction cascades for HR induction (Fig. 9). We speculate that signaling leads to expression of Eth-dependent genes for plant growth and SA- or JA-dependent genes for plant defense. These hypotheses are the subject of ongoing experiments in our laboratory by undertaking the investigation of an unknown SSB<sub>x</sub>-interacting protein in plants.

Supporting Information

Figure S1 Comparison of single-stranded DNA-binding proteins in *Xanthomonas* species and other prokaryotes by multiple sequence alignment. The sequences within the black-dashed line rectangle represent conserved region in *Xanthomonas* but variable in other prokaryotes. Protein accession numbers are indicated. The abbreviations are as follows: Xoc, *X. oryzae* pv. *oryzicola*; Xos, *X. oryzae* pv. *oryzae*; Xcv, *X. campestris* pv. *vesicatoria*; Xcc, *X. campestris* pv. *campestris*; Rs, *Ralstonia solanacearum*; Yo, *Yersinia aldovae*; Ea, *Eschericha amylovora*; Pst, *Pseudomonas syringae* pv. *tomato*; Ee, *Eschericha coli*, and Xf, *Xylella fastidiosa*. (TIFF)

Figure S2 Phylogenetic analysis of SSB proteins in various bacterial species. A neighbor-joining bootstrap tree was derived from the amino acid sequences of SSB proteins using the Vector NTI Align program (http://www.invitrogen.com). Protein accession numbers are indicated after the bacterial species or strain designation. Based on phylogenetic analysis, SSB proteins were classified into one of three groups (I, II and III) for HR induction in host tobacco. (TIFF)

Figure S3 SSB<sub>x</sub>oc binds to single-stranded DNAs in electrophoretic mobility shift assays (EMSA). Randomly synthesized DNA1 and DNA2 (Table S2) were labeled with the Biotin 3'-End DNA Labeling Kit (Thermo, USA). EMSA was performed using protocols supplied with the LightShift Chemiluminescent EMSA Kit (Thermo, USA). Five μg of purified SSB<sub>x</sub>oc protein was mixed with 20 μl of the binding buffer and 20 fmol of biotin-labeled DNA1 (left panel) or DNA2 (right panel); in competition assays (lanes marked with*), labeled DNA was mixed with a 200-fold molar excess of unlabelled DNA1 or DNA2. The mixtures were incubated at room temperature for 20 min. Samples were then loaded on 5% polyacrylamide gels in 0.5X TBE buffer (pH 8.3). Gels were transferred to Hybond N+ membranes (Amersham, Pharmacia), and signals were detected by chemiluminescence according to the manufacturer's instructions. The experiment was repeated twice and similar results were obtained. Lanes that are labeled (−) do not contain SSB<sub>x</sub>oc and DNA. The middle lane in each panel clearly shows the retardation of DNA mobility due to SSB<sub>x</sub>oc binding. (TIFF)

Table S1 Strains and plasmids used in this study. (DOC)

Table S2 Primers used in this study. (DOC)

Table S3 Amino acid identity between SSB<sub>x</sub> in *X. oryzae* pv. *oryzicola* RS105 and homologues in other bacteria. (DOC)

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Author Contributions

Conceived and designed the experiments: HZ GC. Performed the experiments: YL WM YC LZ. Analyzed the data: YL WM HZ. Contributed reagents/materials/analysis tools: LZ WM YL. Wrote the paper: YL MZ GC.

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