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Three sorghum serpin recombinant proteins inhibit midgut trypsin activity and growth of corn earworm

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ABSTRACT

The sorghum (*Sorghum bicolor*) genome contains a number of putative serpins (serine protease inhibitors) that are expressed under varying conditions, but little is known about their biological function. One of the sorghum serpin genes encodes a protein that contains reactive center residues Leu-Arg-X (X = small residue), called a LR serpin. Similar plant-derived LR serpin proteins can inactivate mammalian trypsins and have activity against insect trypsins. In this study the sorghum LR serpin, and two non-LR sorghum serpins, which were expressed in *Escherichia coli* and purified using immobilized metal-affinity chromatography, were shown to inhibit *in vitro* trypsin activity from larval midgut extract of corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*). Each serpin was added individually to sorghum leaf insect diet that was fed to corn earworm and fall armyworm larvae. Statistically significant reductions (30–53%) in the mean weight of corn earworm larvae, but not for fall armyworm larvae, were found in the larvae feeding on diet containing each of the serpins compared to the mean weight of those feeding on control diet. These studies suggest that the sorghum serpin genes could be utilized for corn earworm larvae resistance in sorghum breeding but fall armyworm larvae have compensatory mechanisms to counter the tested sorghum serpins.

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1. Introduction

Crop losses due to insect damage cost billions of dollars every year (Zalucki et al., 2012). Abiotic stress and/or insect damage to grain crops such as rice (*Oryza sativa*), maize (*Zea mays*), sorghum and wheat (*Triticum aestivum*) can also facilitate the growth of molds that produce toxins that are harmful to humans and livestock. Genetically modified plants expressing endotoxins from *Bacillus thuringiensis* (Bt) reduce damage of insect pests, lower pesticide usage and promote the activity of herbivore predators (Lu et al., 2012). Most farmers are pleased with the performance of Bt crops and yields from Bt corn, which are often better than from corresponding non-Bt corn (Carpenter, 2010; Edgerton et al., 2012). However, insect pest populations are developing resistance to the transgenic endotoxins (Tabashnik et al., 2013). Therefore, alternatives to the *B. thuringiensis* endotoxins need to be discovered for continued control of insect populations. Additionally, some markets find transgenic corn undesirable, so there is a need to determine natural resistance mechanisms in order to assist breeders in the development of more insect resistant lines.

Plant derived protease inhibitors are one source of potential resistance molecules that have been studied for many years (Habib and Fazili, 2007). Several different classes of protease inhibitors are produced by plants, such as serpins, trypsin/α-amylase inhibitors, Kunitz-type inhibitors and cystatins (Habib and Fazili, 2007). Inhibition of insect proteases reduces the availability of amino acids that are necessary for continued growth of the herbivore. Insects typically adapt to plant protease inhibitors by expressing new proteases that are insensitive to the inhibitors (de Oliveira et al., 2013), but some protease inhibitors may not trigger this response (Srinivasan et al., 2005). Discovery of additional protease inhibitors that act by novel means and/or do not induce insensitive insect proteases, particularly if they are from crop plants, would be advantageous in developing more insect resistant crops.

Serpins are a superfamily of large (340–440 amino acids in length) irreversible protease inhibitors, predominantly serine protease inhibitors, that are present in most biological organisms (Law et al., 2006; Fluhr et al., 2012). While many human serpins have been well characterized and display a variety of functions (such as in mammalian blood coagulation), few plant serpins have been studied in detail. A

Abbreviations: AtSerpin1, *Arabidopsis thaliana* serpin 1; BSA, bovine serum albumin; Bt, *Bacillus thuringiensis*; IMAC, immobilized metal-affinity chromatography; LR serpin, serpin with leucine and arginine at positions P2 and P1 of the reactive center loop; RCL, reactive center loop; Sbser, *Sorghum bicolor* serpin.

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few human serpins do not inhibit proteases, yet have clearly defined functions, such as hormone transporters and molecular chaperones (Law et al., 2006). A number of different serpins are also produced by most plant species, but the targets of these serpins are unknown in most cases. AtSerpin1 (locus At1g47710), from *Arabidopsis thaliana*, interacts with at least two different proteases (Vercammen et al., 2006; Lampl et al., 2010). In leaves, AtSerpin1 inhibited the pro-death cysteine protease, RD21, when released into the cytosol under pathogen induced stress (Lampl et al., 2013).

AtSerpin1 is a "LR serpin", characterized by a Leu-Arg-X (X = Ser, Cys, Ala or Gly) motif in the reactive center loop (RCL) at positions P2, *P*1 and *P*1′, respectively, where the target protease cuts between *P*1 and *P*1′ (Roberts and Hejgaard, 2008). LR serpins are potent inhibitors of trypsin (Dahl et al., 1996). AtSerpin1 recombinant protein inhibited trypsin and chymotrypsin activities of the midgut from a number of insects and also inhibited the growth of the cotton leaf worm (*Spodoptera littoralis*) when added to insect diet (Alvarez-Alfageme et al., 2011). LR serpins are present throughout the plant kingdom. Sorghum contains a number of putative serpins, but only one LR serpin, encoded by gene Sb01g014740 (Paterson et al., 2009). The sorghum LR serpin (named Sbser1 for this study), has the potential to be utilized as an insect resistance gene. The Sbser1 gene was cloned and expressed using *E. coli* to study its *in vitro* activity against insect midgut trypsin activity and *in vivo* effects when included in sorghum leaf based diet fed to larvae. Two other sorghum non-LR serpins were also expressed *in vitro* for comparison to Sbser1.

2. Materials and methods

2.1. In silico structural modeling and overlay analysis

A multiple sequence alignment with primary protein sequences of the sorghum serpin using Clustal Omega identified the level of overall homology and conservation of *P*1-*P*1′ cleavage site with other serpins. Initial modelled tertiary structures of sorghum serpins were generated using SWISS-MODEL which uses BLAST and HHblits to identify templates and to obtain target-template alignments (Remmert et al., 2012; Biasini et al., 2014). The target/template alignment was used as an input for generating an all-atom model for the target sequence using ProMod-(3.7) (Sali and Blundell, 1993). Global model quality estimation scores obtained for the models for target template alignment were close to one for the three sorghum serpins, indicating a high reliability. A sequence identity of >50% was observed with *Arabidopsis thaliana* AtSerpin1 for each sorghum sequence, although the template modeling score was best for Sbser2. Thus, all three sorghum serpin models were prepared using PDB:3LE2 of *Arabidopsis thaliana* AtSerpin1 (Lampl et al., 2010) as a template which showed maximum coverage percentage and identity. The models were further refined and checked for Ramachandran plot values (92.8%, 93.9% and 89.5% for Sbser1, Sbser2 and Sbser2, respectively) and validated using Procheck. Percentage similarty between alpha-1-antitrypsin (human) and sorghum serpin, was assessed by pairwise alignment using the Needleman-Wunsch algorithm. Sorghum serpin models were superimposed with alpha-1-antitrypsin (1OO8, human) using PyMol.

2.2. Construction of expression vectors

Sorghum seed (BTx623) was obtained from the USDA ARS Plant Genetic Resources Conservation Unit in Griffin, Georgia and grown in pots using soil and fertilizer composition number 3 in a plant growth room as described for maize (Dowd et al., 2007). The serpin coding sequences of Sbser1 (GenBank NC_012870.1) and Sbser3 (GenBankNC_012874.1) are interrupted by a single ~1000 base pair intron. Thus, Sbser1 and Sbser3 coding sequences were PCR amplified from BTx623 sorghum leaf genomic DNA as two fragments that were subsequently joined using Gibson Master Assembly Mix (New England Biolabs, Ipswich, MA). The serpin coding sequence of Sbser2 (GenBank XM_002466778.1) was amplified from BTx623 sorghum leaf cDNA. Each serpin coding sequence was cloned into pET-28b vector for expression of an N-terminal hexahistidine fusion protein in Acella *E. coli* (EdgeBio, Gaithersburg, MD for Sbser2 and Sbser3) or Rosetta 2 (DE3) *E. coli* (EMD Millipore, Darmstadt, Germany for Sbser1). The correct coding sequence of each serpin clone was verified using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, *CA*).

2.3. Serpin expression

Preliminary experiments determined each serpin was expressed as insoluble inclusion bodies after 18–24 h at 37 °C in MagicMedia (Life Technologies, Carlsbad, *CA*). Induced bacteria cells from 50 ml of culture were suspended in 5–7 ml of 100 mM sodium phosphate, pH 7.0, after removal of spent media. Lysozyme (1 mg ml^{-1}), benzonase (25 units ml^{-1}) and EDTA (final concentration at 10 mM) were added to the suspension and incubated for 1 h at 37 °C. The suspension was sonicated three times for 30 s, and placed on ice briefly in between each sonication. The suspension was centrifuged for 10 min at 2655 g. The supernatant was removed and the pellet was suspended in 25 ml of inclusion body wash (0.5% Triton × 100, 50 mM Tris HCl pH 8.0, 10 mM EDTA, 100 mM sodium chloride). Sonication and centrifugation, as described above, was repeated until the supernatant was clear. Inclusion bodies were stored in inclusion body wash at 4 °C.

Inclusion bodies were denatured in 10–15 ml of 6 M guanidine HCl, 50 mM Tris HCl pH 7.8 at room temperature with gentle rocking. The denatured serpin was slowly dripped into 1.6 l of refolding buffer (50 mM Tris HCl, pH 8.0, with 50 mM NaCl) at 4 °C. Refolded serpins were concentrated and purified by immobilized metal-affinity chromatography (IMAC). An AKTA purifier (GE Healthcare, Waukesha, WI, USA), equipped with a superloop, was used to load 150 ml of serpin onto a HisTrap HP (1 ml) column (GE Healthcare). The column was then washed with 2 ml IMAC binding buffer (50 mM Tris-Cl (pH 8.0), 10 mM NaCl) to remove unbound proteins. Bound serpins were then eluted with 5 ml IMAC elution buffer (50 mM Tris-Cl (pH 8.0), 0.5 M NaCl, 0.3 M imidazole). Elution fractions of 0.5 ml were collected and the serpin containing fraction was identified by protein assay. The eluted serpin, as well as an aliquot of elution buffer, was dialyzed against 10 mM sodium phosphate pH 8.0 for 2–3 h at 25 °C using a Slide-A-Lyzer MINI dialysis cassette (10,000 molecular weight cutoff, Pierce Biotechnology, Rockford, IL) with shaking at 200 RPM. The dialysis buffer was replaced and the dialysis cassettes were incubated overnight at 4 °C. Purified Sbser2 and Sbser3 were analyzed by SDS 15% (*w*/*v*) polyacrylamide gel electrophoresis (Schägger, 2006) and the gel stained with Bio-Safe Coomassie stain (Bio-Rad, Hercules, *CA*) according to the manufacturer's instructions. The proteins were visualized using a Molecular Imager ChemiDoc XRS + (Bio-Rad).

2.4. Gel-based trypsin binding assay

Sbser1, eluted from IMAC chromatography, was desalted into anion exchange binding buffer (20 mM Tris-Cl pH 7.5) using a gel filtration column (HiTrap Desalting, GE Healthcare). Desalted Sbser1 was loaded on an anion exchange column (HiTrap Q XL (1 ml), GE Healthcare). The protein was eluted from the column using a linear salt gradient (A, 20 mM Tris-Cl pH 7.5 and B, 20 mM Tris-Cl pH 7.5 with 0.5 M NaCl), from 0% B to 100% B in 10 min, with a flow rate of 1 ml per min. The major peak was collected as a single 0.5 mL fraction. To visualize serpin-trypsin complexes, 1 μg of Sbser1 in 20 mM Tris-Cl pH 7.5 and ~0.15 M NaCl was incubated with 0.5 μg porcine trypsin (Sigma-Aldrich, St. Louis, MO) for 5 min at 22 °C. Reactions were then boiled in SDS-PAGE sample buffer and loaded onto a Criterion TGX 12% gel (Bio-Rad). Proteins were separated for 25 min at 250 V and visualized by staining with Oriole florescent stain (Bio-Rad).

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