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ORIGINAL PAPER

Characterization of an Insecticidal Protein from *Withania somnifera* Against Lepidopteran and Hemipteran Pest

Blessan Santhosh George[1] · S. Silambarasan[2] · K. Senthil[2] · John Prasanth Jacob[2] · Modhumita Ghosh Dasgupta[1]

Abstract

Lectins are carbohydrate-binding proteins with wide array of functions including plant defense against pathogens and insect pests. In the present study, a putative mannose-binding lectin (*WsMBP1*) of 1124 bp was isolated from leaves of *Withania somnifera.* The gene was expressed in *E. coli,* and the recombinant WsMBP1 with a predicted molecular weight of 31 kDa was tested for its insecticidal properties against *Hyblaea puera* (Lepidoptera: Hyblaeidae) and *Probergrothius sanguinolens* (Hemiptera: Pyrrhocoridae). Delay in growth and metamorphosis, decreased larval body mass and increased mortality was recorded in recombinant WsMBP1-fed larvae. Histological studies on the midgut of lectin-treated insects showed disrupted and diffused secretory cells surrounding the gut lumen in larvae of *H. puera* and *P. sanguinolens,* implicating its role in disruption of the digestive process and nutrient assimilation in the studied insect pests. The present study indicates that *WsMBP1* can act as a potential gene resource in future transformation programs for incorporating insect pest tolerance in susceptible plant genotypes.

Keywords Insecticidal lectin · Mannose binding · Secretory cells · Teak defoliator

Introduction

Plants possess complex defense mechanisms to counter attacks by pathogens and parasites, ranging from viruses to animal predators. Both mechanical and chemical mechanisms have evolved, which allow plants to coexist in an ecosystem which are also inhabited by their potential foes [55, 59]. Defense response of plants varies depending on the nature of the invading organisms [24].

Lectins are ubiquitously distributed in diverse life forms. They are carbohydrate-binding proteins capable of specific recognition and reversible binding to carbohydrates and sugar-containing substances, without altering covalent structure of any glycosyl ligands. They possess two or more carbohydrate-binding sites [27] and display an enormous diversity in their sequence, biological activity and mono- or oligosaccharide specificity, in addition to structural versatility [41, 50]. Plant lectins are classified based on sequence similarities and evolutionary relationships [60], and presently twelve distinct lectin families are recognized [59]. Each lectin domain is characterized by the overall fold with one or more carbohydrate-binding sites. Lectins with different domain specificity are reported from soybean, rice and Arabidopsis, and all major lectin superfamilies are present in each species [22].

The entomotoxicity of plant lectins has been extensively reviewed [4, 26, 28, 61]. Insecticidal lectins are predominantly resistant to proteolytic degradation by digestive enzymes of insect, and their toxic nature is attributed to interaction with insect glycoprotein or glycan structures, which interfere with major physiological processes [28, 64]. They bind to brush-border membrane of the epithelial cells of insect intestine or to peritrophic membrane (PM) [9, 41]. In case of insects lacking a PM, the insecticidal effect of lectins is caused by their direct interaction with glycoconjugates present on the cell epithelium [43]. Lectins

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✉ Modhumita Ghosh Dasgupta
ghoshm@icfre.org; gmodhumita@gmail.com

[1] Division of Plant Biotechnology and Cytogenetics, Institute of Forest Genetics and Tree Breeding, RS Puram, Coimbatore, Tamil Nadu 641002, India

[2] Plant Protection Division, Institute of Forest Genetics and Tree Breeding, RS Puram, Coimbatore, Tamil Nadu 641002, India

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are reported to cause mortality, delay in development and/or adult emergence, reduced fecundity, growth and development in insect pests [28, 32].

Mannose-binding lectins are widely distributed in plants and possess defense role against microbes and insect predators [6]. The first monocot mannose lectin with insecticidal properties was reported from snowdrop (*Galanthus nivalis*) bulb and designated as GNA [56]. Subsequently, several monocot mannose-binding lectins were characterized from Alliaceae, Amaryllidaceae, Areaceae, Bromeliaceae, Iridaceae, Liliaceae and Orchidaceae [60]. A plethora of GNA-related lectins was shown to impart insecticidal actions against different groups of insect pests including jackbean concanavalin A (ConA) [48]; *Pinnelia ternata* agglutinin (PTA; [19, 40]), *Listera ovata* agglutinin (LOA; [29]), *Allium cepa* agglutinin (ACA; [18]); *Allium sativum* leaf agglutinin (ASAL; [42, 46, 51, 64]); *Allium porrum* lectin [45]; *Colacasia esculenta* [10]; *Diascorea batatas* (DB1) [39]; *Colocasia esculenta* and *Diffenbachia sequina* [42]. The mannose-binding lectin Orysata isolated from *Oryza sativa* was reported to have insecticidal activity against armyworm, green peach and pea aphids in both in vitro artificial diet [2] and *in planta* validation [28]. In a recent study, a mannose-binding lectin was isolated from bulbs of *Phycella australis* and named *Phycella australis* agglutinin (PAA) and their toxic, antireproductive and feeding deterrent nature was demonstrated against aphids [65]. In the present study, a lectin with predicted mannose-binding domain was isolated from leaf tissue of *Withania somnifera* and the insecticidal property of the recombinant protein was demonstrated against two insect pests.

Materials and Methods

Plant Material

Seeds of *W. somnifera* were provided by Regional Forest Research Institute, Andhra Pradesh Forest Department, Rajahmundry, Andhra Pradesh, India. Seeds were surface sterilized and germinated in ½ MS media (HiMedia, India). The axillary shoots emerging from the plantlets were used as explants for in vitro propagation and subsequent gene isolation studies.

Isolation of *WsMBL1* Gene

Total RNA was isolated from leaves of in vitro grown *W. somnifera* plants that were treated with 5 mM salicylic acid [16] using plant total RNA extraction spin kit (Chromous Biotech, India) as per the protocol described by the manufacturer. First-strand cDNA was synthesized from DNAse-treated total leaf RNA using AMV reverse transcriptase and oligo dT-3 sites adaptor primer provided in 3′-Full RACE Core Kit (Takara Bio Inc., Japan). 3′ RACE was carried out using forward primer MBLLP2 designed based on the leaf transcriptome data of *W. somnifera* [16]. Nested PCRs were performed with MBLLP2 and WSMBLF3. The PCR program had an initial denaturation at 94 °C for 5 min followed by 30 cycles with each cycle comprising denaturation (94 °C, 1 min), annealing period of 30 s and extension (72 °C, 2 min). Final extension at 72 °C was performed for 7 min.

Subsequently, 5′ RACE was carried out using FirstChoice RLM-RACE Kit (Ambion, CA, USA) following the manufacturer's instructions. WSMBLR2 and WSMBLR3 were the reverse primers used for carrying out the outer PCR, while WSMBLR3 and WSMBLR4 were used for the inner PCR (Supplementary Table 1). Gene-specific primers, WSCDS F1: ATGTCTCCTTCATGGACTACTACAC and WSCDS R: TCATATCCTGCTGTAAGTTACCCTCCA, were designed and synthesized to amplify the complete coding domain sequence (CDS). Amplified products were cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning Kit (Fermentas, Hanover, MD, USA) following the manufacturer's instructions and sequenced.

In Silico Analysis of Nucleotide Sequence

Sequence similarity was determined using BLASTN program at NCBI [3], optimized for high similar sequences (megablast). Open reading frame analysis of CDS was carried out using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). Molecular weight, theoretical pH and disulfide bond formation in the putative polypeptide were predicted using software available online at EXPASY and DiANNA (http://web.expasy.org/cgibin/compute_pi/pi_tool and http://bioinformatics.bc.edu/clotelab/DiANNA). Analysis of putative protein domains was carried out using Conserved Domain Database (www.ncbi.nlm.nih.gov/cdd) and InterPro (www.ebi.ac.uk/Tools/pfa/iprscan). Post-translation modification processes likely to occur in the theoretical nascent protein were also predicted. Signal peptide cleavage site was predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP), while analysis for the presence of potential glycosylation and phosphorylation sites was carried out using NetNGlyc1.0 (www.cbs.dtu.dk/services/NetNGlyc) and NetPhos2.0 (www.cbs.dtu.dk/services/ NetPhos), respectively. Potential transmembrane segments were identified (www.sbc.su.se/~miklos/DAS), and subcellular localization of the theoretical protein was predicted using Plant-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant) and SCLpred [35]. In silico determination of potential protein allergenicity was conducted using EVALLER web server (http://www.slv.se/en-gb/Group1/Food-Safety/e-Testing-of-proteinallergenicity). Additionally, secondary structure

analysis of the sequence was carried out using SOPMA program (http://npsa-pbil.ibcp.fr/cgibin/secpred_sopma).

Phylogenetic Analysis

The evolutionary relationship of the translated gene product with other full-length plant lectins and glycoproteins was conducted using MEGA version 5.05 [54]. The molecular distances of the aligned sequences were calculated according to the parameter of p-distance, and the phylogenetic tree was generated using the rooted NJ method. Pair-wise deletions were used to deal with gaps, and relative level of support for the tree topology was analyzed by determining the bootstrap values from 1000 replicates. *Bos taurus* albumin was used as an outgroup.

Expression of *WsMBP1* in *E. coli*

pET 28a (+) expression vector (Novagen, EMD Biosciences, San Diego, CA) was used for expression of *WsMBP1* in bacterial system. For cloning experiments, the coding sequence devoid of signal peptide was amplified using the primers WSMBL_sP_F: TTAACGGTTCATATGCAAGTTCCA GATGAAAACA and WSMBL_Not_R: TAAGCTTGTGCG GCCGCTATCCTGCTGTAAGTTACCCTCCA and was directionally cloned in pET28a using the primer pairs WS_ LEC_Sac_FWD: GGAATTCCGAGCTCATGTCTCCTT CATGGACTACTACAC and WS_LEC_Hind_R: GACCGC AAGCTTTCATATCCTGCTGTAAGTTACCCTCCA. The construct WsMBP1:pET28a was transformed into competent BL21(DE3) *E. coli* cells (Novagen, Merck KGaA, Darmstadt, Germany), and recombinant protein was expressed by induction with 1 mM of IPTG at 18 °C for 3 h.

Protein Purification and Analysis

Recombinant lectin was predominantly present in the inclusion bodies (IB). The bacterial pellet was suspended in lysis buffer (50 mM Tris–HCl, 01 mM EDTA, 5% glycerol, 01 mM DTT and 01 M NaCl; pH = 70) containing 1 mM PMSF and 1 mg/ml lysozyme followed by sonication and centrifugation at 15,000 rpm for 15 min at 4 °C to isolate the purified IB. Subsequently, purified IB was solubilized in 2% sarkosyl (HiMedia, Mumbai, India), 8 M urea (Sigma, Sigma-Aldrich, St Louis, MO, USA), 6 M guanidinium-HCl (Qiagen, Hilden, Germany) and 0.5% SDS (HiMedia, Mumbai, India) to determine the most suitable solubilizing agent. The solubilized fraction was clarified by centrifuging at 12,000 rpm for 20 min and dialyzed overnight against Tris-buffer, pH = 70, for facilitating protein re-folding. The solubilized re-folded protein was initially concentrated by precipitating with 30% saturated ammonium sulfate and passed through Sephacryl S-100 column (Sigma, Sigma-Aldrich, St Louis, MO, USA) previously equilibrated and eluted with 100 mM Tris–HCl, pH 7.0. Fraction was collected, and absorbance measured at 280 nm, pooled and passed through ConA-Sepharose column (Sigma, Sigma-Aldrich, St Louis, MO, USA). After application of the sample, protein was eluted with 200 mM methyl a-D mannopyranoside (Sigma, Sigma-Aldrich, St Louis, MO, USA). The eluted protein was resolved on a 12% SDS-PAGE, and the induced protein was excised and placed in an activated dialysis tubing cellulose membrane (33 mm × 21 mm; Sigma-Aldrich, St. Louis, MO, USA) containing 0.1 M phosphate buffer (pH = 8.0) and placed in a submarine electrophoretic unit filled with 1× Tris–glycine buffer. Electrophoresis was carried out at 80 V for ~ 3 h at 4 °C, and subsequent to complete elution, the protein was recovered, resolved in 12% SDS-PAGE, silver stained and confirmed by LC–MS/MS analysis. Inclusion bodies obtained from bacterial cells harboring empty pET28a vector were also isolated, solubilized and used as control sample for subsequent experiments.

Hemagglutination assay of recombinant WsMBP1 was performed by twofold serial dilution method. The assay was carried out using rabbit, mice and rat erythrocytes using 2–4% red blood cell suspension in 0.9% saline. Fifty microliters of serial twofold dilutions of sarkosyl-solubilized purified inclusion bodies containing the recombinant lectin at the concentration of 1 µg/100 µl was mixed with equal volume of erythrocyte suspension, and the plates were incubated at room temperature. Purified inclusion body devoid of the lectin obtained from vector-less bacterial cells was kept as the control. Results were documented after 1 h of incubation. The experiment was conducted at KMCH College of Pharmacy, Coimbatore, India. KMCH College of Pharmacy has a centralized animal house facility approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (Approval No: 685/PO/Re/S/2002/CPCSEA).

Functionality Analysis of the Recombinant Lectin for Insecticidal Properties

Toxicity of the recombinant lectin was tested against two insect pests including *Hyblaea puera* (Lepidoptera: Hyblaeidae) and *Probergrothius sanguinolens* (Hemiptera: Pyrrhocoridae). Insects were selected based on their feeding habits wherein *H. puera* is a chewing insect pest, while *P. sanguinolens* is a sap feeder. Larvae of *Hyblaea puera*, a defoliator and prominent pest of *Tectona grandis* were collected from the field and reared on teak leaves in the laboratory at room temperature. Adults of *Probergrothius sanguinolens* (seed feeder) were reared on a sand bed in plastic troughs provided with seeds of *Sterculia foetida* and water.

Bioassay Against Insect Pests

Bioassay was conducted on *H. puera* larvae on their second day after hatching. Five numbers of even aged larvae in four batches were introduced to fresh teak leaves applied with the recombinant lectin suspension (~ 10 µg per leaf) and allowed to feed. Equal number of larvae fed on leaves smeared with purified solubilized inclusion bodies obtained from empty pET28a vector was used as control. Percentage of insect survival/mortality, weight gain, number of days taken to reach pupal stage and percent adult emergence was documented for *H. puera*.

In *P. sanguinolens*, 50 µl of protein solution (50 µg/ml) was mixed with equal volume of sugar solution (02 g/ml) and kept in tube for insect feeding. Three batches of five 0-day second instar nymphs were assessed till they molted to third instar stage. The same number of larvae fed on lectin devoid IB was used as control. Percent mortality, number of days taken for molting to next instar and percentage successful molting were recorded. Both the experiments were performed in two independent replicates.

Histological Studies

Histological studies of insect gut were conducted in control and lectin-treated *H. puera* and *P. sanguinolens.* Two insects from each batch of *H. puera* [7] and *P. sanguinolens* [6] were dissected out in insect Ringer's solution and fixed in Carnoy's solution. Wax blocks were subsequently prepared, and 7-micron-thick sections were taken using a rotary microtome (Micros, Austria). The sections were mounted on slides and stained with Ehrlich's hematoxylin followed by counterstaining with eosin. The best differentiated sections were further analyzed.

Results

Cloning of Full-Length cDNA of *WsMBP1* and In Silico Sequence Analysis

The full-length gene designated as *WsMBP1* encompassed a 200-bp 3′ UTR, 30-bp 5′ UTR and 894-bp CDS (GenBank Accession No KC329532). The sequence showed a significant similarity to mannose-binding lectin from *Capsicum annum*, *CaMBL1* ($E = 00$; identities = 88%), sugar-binding lectin from *Arabidopsis thaliana* ($E = 3e{-}65$) and *A. lyrata* and SIEP1L protein from *Beta vulgaris* ($E = 5e{-}97$). The CDS of *WsMBP1* encoded a putative protein of 297 amino acids with a predicted molecular weight of 33.83 kDa and a theoretical isoelectric point (pI) of 9.49. Disulfide bond formation was predicted between the 22nd and 64th cysteine residues of the putative protein. Signal peptide prediction tool, SignalP 4.0, revealed a signal peptide cleavage site between 24th and 25th residues in the sequence. Analysis of protein domains was carried out using Conserved Domain Database and InterPro, which detected a bulb-type mannose-specific lectin domain extending from 45th to 162nd residues. Potential transmembrane segments were identified in the region flanking 8th and 22nd amino acids. Analysis for the presence of potential glycosylation site in the putative protein revealed four predicted sites for *N*-glycosylation with three positions crossing the threshold value. Further, analysis using NetPhos2.0 indicated that the deduced protein sequence had fifteen predicted phosphorylation sites. The predictions were made for serine and threonine residues with each residue accounting for ten and five predicted sites, respectively, while no tyrosine residues in the putative amino acid sequence had a phosphorylation potential above the set threshold. Subcellular localization prediction with SCLpred and Plant-PLoc designated the putative lectin to be secretory in nature with medium confidence level.

Multiple sequence alignment of B-lectin domain sequence of *WsMBP1* with similar domains in mannose-binding lectins isolated from *C. annuum* and *G. nivalis* revealed that these domains were identical, but deviated from the conventional QXDXNXVXY consensus sequence motif reported for monocot mannose-binding lectins as in *G. nivalis* agglutinin (Fig. 1).

In silico test for protein allergenicity using EVALLER generated five best matching peptides (FLAPs) with low degree of similarity, and the predicted protein was categorized as "presumably not an allergen" with an uncertainty probability of 5.6%. The secondary structure analysis of the lectin with SOPMA program revealed that the protein is predominated by β-sheets other than the random coil. The putative peptide contained approximately 12% of α-helix, 38% of β-sheet, 9% of β-turn and 41% of random coil.

The evolutionary relationship of the translated *WsMBP1* with other full-length plant lectins and glycoproteins was conducted using MEGA. The results showed that *WsMBP1* was closely related to mannose-binding lectin from *C. annum*. The phylogenetic analysis also revealed that the translated *WsMBP1* was related to epidermis-specific secreted glycoproteins across different families in plant kingdom (Fig. 2).

Expression and Purification of Recombinant Lectin

The optimum expression of *WsMBP1* in bacterial system was achieved when the CDS devoid of signal peptide was cloned and induced with 1 mM IPTG and incubated for 3 h at 18 °C. The approximate size of the recombinant lectin was ~ 31 kDa (Fig. 3).

Bacterial inclusion bodies were solubilized in different detergents and chaotropic agents. It was observed that

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1MSA_A      DNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDK-------------------PIW
WSMBP1      -------------------RVLSVFTNPFQLCFYNTTPNAWTLALRMGTVRSESLMRWVW
CAMBL1      -------------------RVLRIATTPFQLCFYNTTPNAWTLALRMGTVRSESLMRWVW

1MSA_A      ATNTGG-LSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVI--YG
WSMBP1      EANRGNPVKENATFSLGTDGNLVLAEANGRIVWQTNTANKGVTGFKLLPNGNMVLHDSKG
CAMBL1      EANRGNPVKENATFTFGTDGNLVLADADGRIAWQTNTANKGVTGFKLLPNGNMVLHDSKG

1MSA_A      TDRWATGTHTG
WSMBP1      KFVWQSFNHP-
CAMBL1      KFLWQSFNYP-
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Fig. 1 Multiple alignment of the B-lectin domain sequence of predicted WsMBP1 with other mannose-binding lectins. 1MSA_A: B-lectin domain sequence of GNA (*Galanthus nivalis* agglutinin) A chain. CAMBL1: *Capsicum annuum* mannose-binding lectin. Residues highlighted in green denote the mannose-binding sites in 1 MSA_A, while those that are boxed correspond to mannose-binding motifs in WSMBP1 and CAMBL1. Residues in red are Man-binding sites conserved across the three aligned sequences, while those in blue are conserved residues but within the domain. Residues represented in yellow are conserved among WSMBP1 and CAMBL1 (Color figure online)

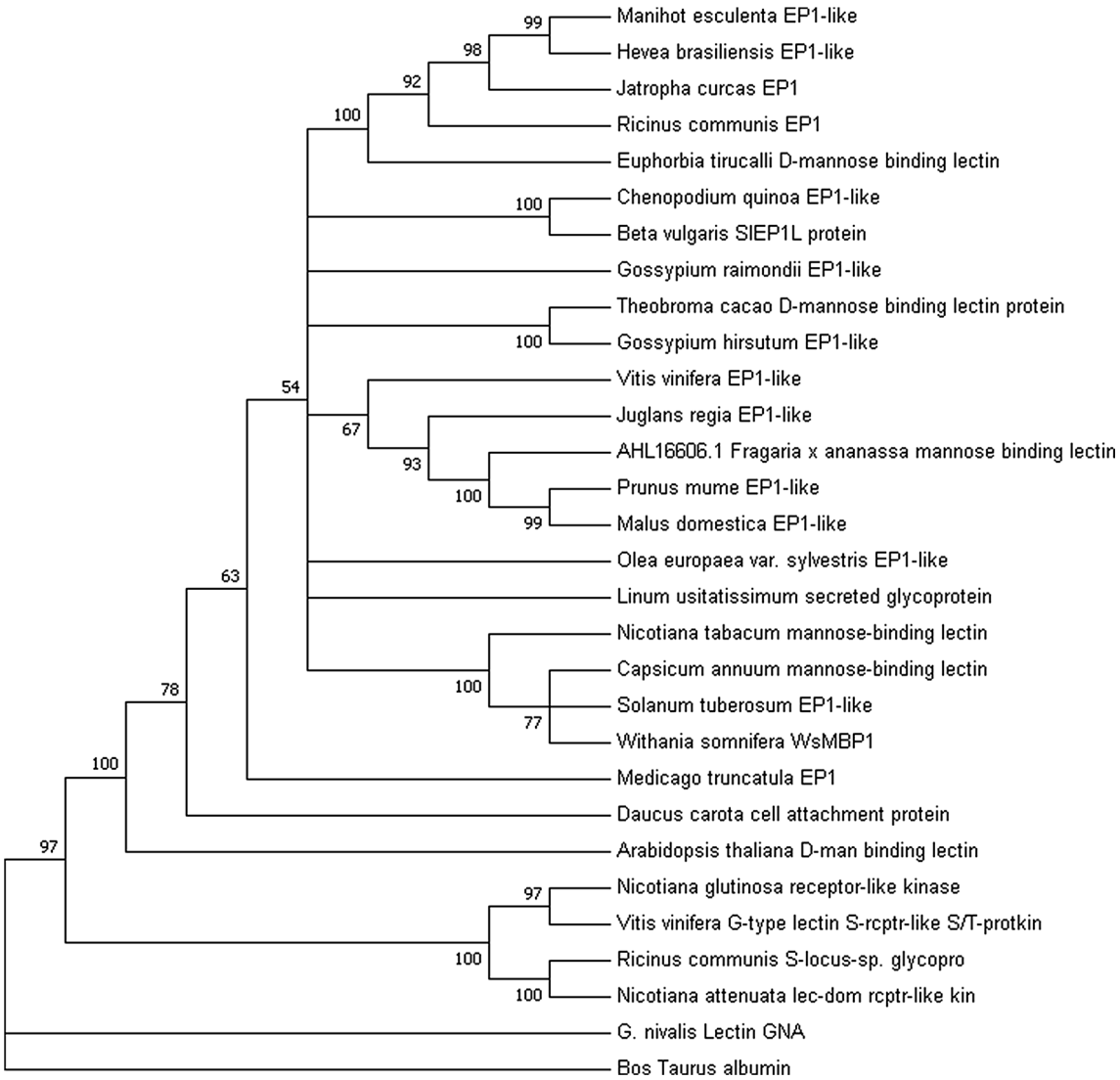

Fig. 2 Evolutionary relationship of the translated *WsMBP1*

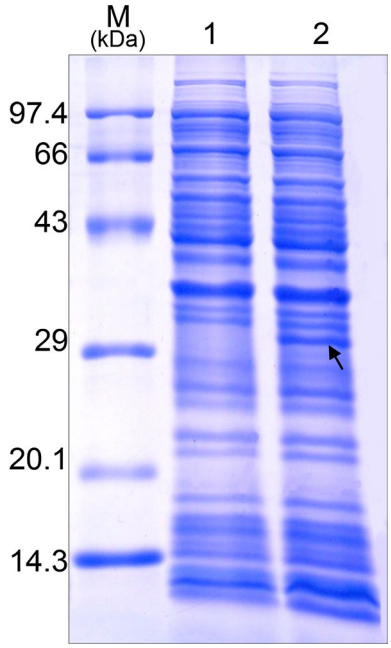

Fig. 3 SDS-PAGE analysis of over-expressed WsMBP1 under optimized conditions. *M* = standard protein molecular weight marker (14.4–97.4 kDa). *Lane 1* IPTG-induced bacterial protein harboring empty pET 28a vector. *Lane 2* IPTG-induced bacterial protein harboring pET-WsMBP1 construct. Approximately, 250 µg of bacterial protein was loaded in each well. Arrow indicates over-expression of the recombinant lectin (~ 31 kDa)

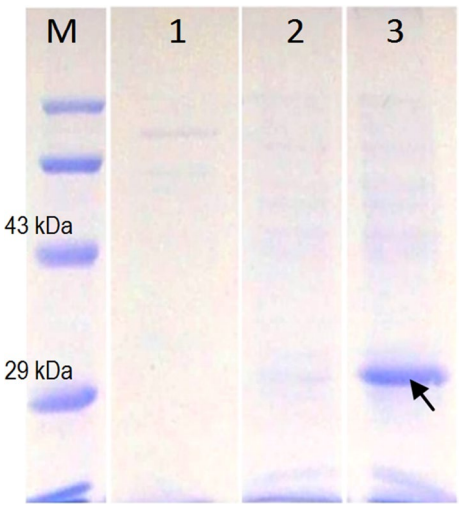

Fig. 4 SDS-PAGE analysis of re-folded inclusion bodies solubilized using different chaotropic agents. Inclusion bodies solubilized with. *M* = standard protein molecular weight marker (14.4–97.4 kDa). *Lane 1* Inclusion bodies solubilized with 6 M guanidinium-HCl. *Lane 2* Inclusion bodies solubilized with 0.5% SDS, and *Lane 3* inclusion bodies solubilized with 2% sarkosyl. Approximately, 50 µg of solubilized protein was loaded in each well. Arrow indicates protein band corresponding to recombinant WsMBP1

the yield of the re-folded protein from the inclusion bodies solubilized using 2% sarkosyl was highest with ~ 40–50% (Fig. 4). The yield of recombinant lectin in solubilized inclusion bodies obtained after dialysis was determined to be 10 µg/ml. Further, purification of the recombinant protein was carried out by gel filtration chromatography and affinity chromatography followed by electroelution, which yielded a single band at ~ 31 kDa (Fig. 5).

LC–MS/MS Analysis of the Over-Expressed Protein

LC–MS/MS data of the recombinant lectin were searched using MASCOT 2.4 against both UniProt/Swiss-Prot and UniProtTrEMBL databases. The TrEMBL results revealed peptide fragment with 4.7% coverage to mannose-binding lectin from *Capsicum annuum,* confirming the protein identity.

Hemagglutination Assay

Hemagglutination assay conducted against erythrocytes from rabbit, rat and mice showed no significant agglutination, revealing that the purified recombinant lectin did not have hemagglutinating property against the tested organisms (data not shown).

Functionality Analysis of the Recombinant Lectin for Insecticidal Properties

Bioassay against *H. puera* larvae revealed 65% mortality in lectin-treated insects, whereas 5% mortality was observed in control insects throughout the life cycle. Weight gain by larvae was also high in control insects (153.7 mg), when compared to treated insects (60.78 mg) (Fig. 6). Pupation of larvae in control set occurred in 14 days, while in lectin-treated larvae, the pupation was significantly delayed and was recorded on 21st day. Metamorphosis from pupae to

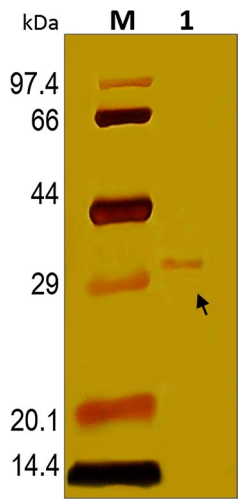

Fig. 5 SDS-PAGE analysis of purified WsMBP1. *M* = standard protein molecular weight marker (14.4–97.4 kDa). *Lane 1* Purified WsMBP1 (~ 2 µg) obtained after electroelution. Arrow indicates protein band corresponding to over-expressed WsMBP1

Fig. 6 Morphological features of recombinant WsMBP1-treated *Hyblaea puera* at pre-pupation larval stage. **a** Insect larvae fed on control sample, **b** insect larvae fed on recombinant protein suspension

adult was not significantly different in insects from both control and treated sets (Table 1). Further, 100% adult emergence was recorded in both the batches.

In *P. sanguinolens,* 96% of nymphs were dead in 7 days of second instar stage, while 53% were dead in control set. Successful molting of 60% was recorded in nymphs of *P. sanguinolens* in control set, while none of insects in treated batch were able to molt to the third instar stage (Table 2). Further, cadavers of the lectin-treated nymphs showed a typical abdominal shrinkage (Fig. 7).

Histological Studies

Histological studies on the midgut of lectin-treated and control insects showed disrupted and diffused secretory cells surrounding the gut lumen in larvae of *H. puera* (Fig. 8a) and *P. sanguinolens* (Fig. 8b). In insects fed with control bacterial protein, intact secretory cells surrounding the gut lumen were recorded (Fig. 8a, b).

Table 2 Effect of recombinant WsMBP1 on metamorphosis and survival of *P. sanguinolens*

Sample	% Mortality	Metamorphosis parameters	
		No. of days taken from second to third instar	% Emergence[a]
Control	60.3 ± 10.3	7.5 ± 1.2	60.0 ± 12.1
Lectin treated	96.7 ± 4.7	7.8 ± 1.5	0

Data are expressed as mean ± SD

[a]Second instar to third instar stage

Discussion

Insects continue to be a major threat to crops worldwide, and increasingly efficient ways to control sap-sucking insect attacks on several important crops are being studied [7]. In recent years, the use of insecticidal lectins in transgenic plants has been considered as a promising strategy in pest management [33], since transgenics expressing Cry toxins from *Bacillus thuringiensis* demonstrated resistance to sap-sucking pests like aphids, thrips and bugs [4, 28, 31]. Ectopic expression of entomotoxic lectins like GNA, ASA and ASAL in diverse crop plants has revealed their toxicity against sap-sucking pests and also pests belonging to homopteran and lepidopteran orders (reviewed by Bakhsha et al. [4]).

Isolation and Characterization of Mannose-Binding Lectin from *W. somnifera* Leaves

Lectins are ubiquitous proteins with varying biological functions. They are capable of recognizing and interacting with specific carbohydrate structures, either originating from the invading organisms or from damaged cell wall structures [26]. Additionally, lectin domains are present in transmembrane proteins that are essentially pattern recognition receptors (PRRs) negotiating plant defense signaling after perception of extracellular signals (viz damage-associated molecular patterns, DAMPs) and thus involved in plant innate immunity.

Table 1 Effect of recombinant WsMBP1 on growth, metamorphosis and survival of *Hyblaea puera*

Sample	% Mortality	Weight gain (mg/larva)[a]	Metamorphosis parameters		
			No. of days taken		% Emergence to adult
			Larvae to pupae	Pupae to adult	
Control	5 ± 2.7	153.7 ± 13.26	14 ± 2.5	3 ± 1.5	100
Lectin treated	65 ± 9.15	60.78 ± 11.46	21±1.5	4 ± 2.6	100

Data are expressed as mean ± SD

[a]Weight gain of larva from introduced larval stage to pre-pupal

Fig. 7 Bioassay of recombinant WsMBP1 against *Probergrothius sanguinolens.* **a** *Probergrothius sanguinolens* nymphs fed on control protein suspension, **b** *P. sanguinolens nymphs* fed on protein suspension containing *WsMBP1.* Arrows indicate shrinkage observed in the abdominal region of lectin-fed nymphs

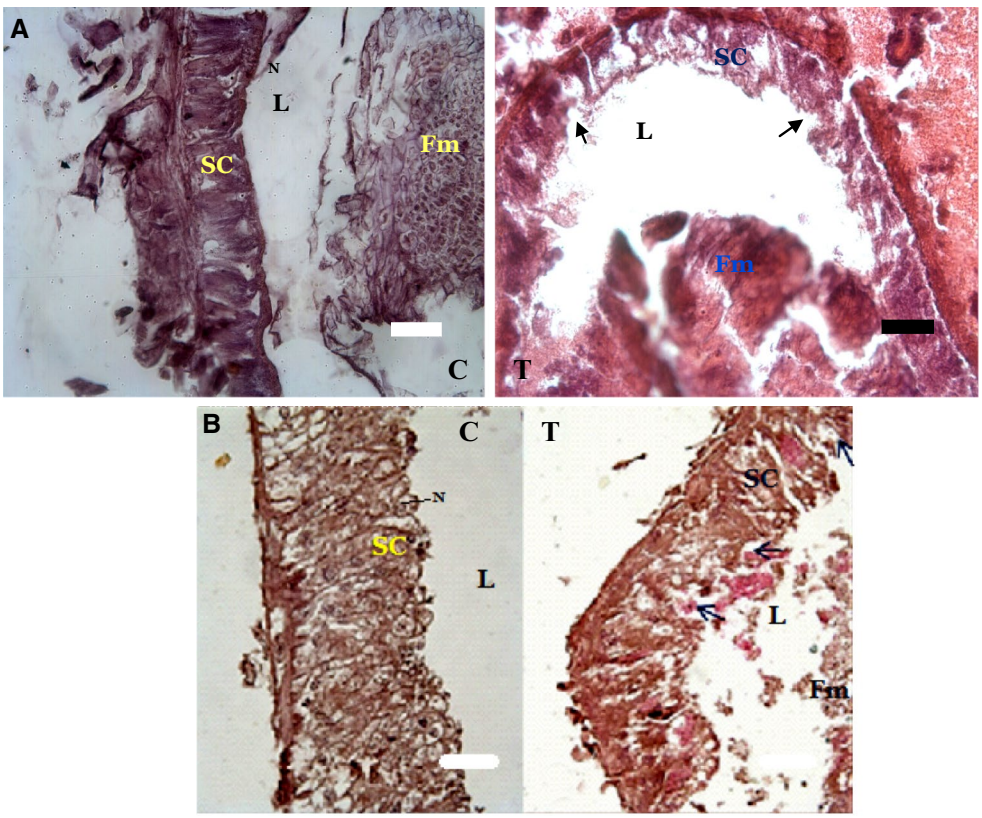

Fig. 8 Histological studies of insect midgut in *Hyblea puera* and *Probergrothius sanguinolens.* C and T in each row are the gut sections of control and recombinant protein suspension-fed insects, respectively. *L* gut lumen, *SC* secretory cells, *Fm* food mass, *N* nucleus. Arrows indicate disrupted and diffused secretory cells surrounding insect gut lumen. Bar represents 20 µm

In the present study, a protein harboring bulb-type mannose-specific lectin domain was isolated from leaves of *W. somnifera*. The complete gene had a size of 1124 bp and consisted of a 894-bp open reading frame, which was highly similar to the *Capsicum annuum* mannose-binding lectin (*CaMBL1*) characterized by Hwang and Hwang [20]. The size of ORF was also in accordance with other mannose-binding lectins reported from Solanaceae species, viz *C. annuum* (*CaMBL1*; GenBank Accession No. GQ2658921) and *N. tabaccum* (Accession No.: JQ0313581).

A signal peptide of 24 amino acid residues was predicted in the translated peptide of WsMBP1 using SignalP online tool. The tripartite structure of the signal peptide is a 8-residue-long (MSPSWTTT) n-region, 12-amino acid-long hydrophobic h-region (LFASLFLFSQIF) and a 4-residue c-region (CCIA). The present result is similar to mannose-binding lectins isolated from pepper and tobacco (GenBank Accession No. GQ265892 and Accession No.: JQ0313581). The signal peptide cleavage site was positioned between 24th and 25th amino acid residue in WsMBP1. Exactly 50% of the predicted N-terminal signal peptide is composed of hydrophobic residues which indicates that WsMBP1 is synthesized on endoplasmic reticulum and follows the secretory pathway. This aspect of subcellular localization was proposed for GNA and other GNA-related mannose-binding lectins like *L. ovata* agglutinin (LOA), *A. sativum agglutinin* I (ASA-I), *Arum maculatum* agglutinin (AMA), *Tulipa lectin* and CaMBL1, the *C. annum* lectin [20, 58, 60].

In silico analysis of the deduced amino acid sequence revealed that WsMBP1 encompassed B-type lectin domain, as found in GNA. GNA-related lectins, as reported from *A. sativum*, *Crocus vernus, Taxus media* and *Zephyrathes grandiflora*, comprise a characteristic β-prism II fold architecture having a threefold symmetry [23, 52, 57]. A threefold internal repeat with a QXDXNXVXY consensus sequence motif is found in these lectins, and this motif is considered to be involved in α-D-mannose recognition [59] in GNA-related lectins. Other monocot mannose-binding lectins show single-residue variation when compared with GNA in one or two of the three conserved motifs, as evident in two lectins isolated from *A. sativum* [51, 52]. However, the described motif was not identified in the deduced amino acid sequence of *WsMBP1* described in the present study. Comparative study with the closely related lectin from *C. annuum* (CaMBL1) revealed that they too lacked the motif sequence described in GNA and related lectins. Hwang and Hwang [20] from their carbohydrate-binding and mutation studies with CaMBL1 concluded that the lectin possessed mannose-binding ability even in the absence of the orthodox sequence motif.

A mannose-binding lectin in *Euphorbia tirucalli* (Euphorbiaceae), identified by gene ontology analysis of lactifer transcriptome, shared domain sequence similarity with WsMBP1 [25]. They also shared similarity to lectin with Apple-like carbohydrate-binding domain identified in *Theobroma cacao* [36] and FaMBL1 from strawberry [17] with minor residual substitutions in the sugar-binding motifs. Presence of sugar-binding domains with high sequence similarity in members of unrelated taxa provides insight in the evolution of the B-lectin domain. These studies indicate that in course of evolution, domains in lectins have undergone dynamic changes, resulting in formation of proteins with diverse sequences, while retaining their binding property.

In silico determination of potential protein allergenicity using EVALLER web server predicted WsMBP1 to be non-allergenic. Mondal et al. [34] had tested the insecticidal *A. sativum* leaf agglutinin (ASLA) against aphids for its allergenicity and reported that ASLA possessed no apparent features of an allergen, as predicted for WsMBP1.

Defense-Related Role of Plant Lectins

The insecticidal property of lectins is well documented against insect pests belonging to both lepidoptera and hemiptera [13, 15, 47]. The entomotoxic effect of GNA administered as artificial diet on Mexican rice borer (*Eoreuma loftini*) was reported by Setamou et al. [49], and it significantly decreased larval survival and adult emergence. Similarly, GNA affected the growth and development of beet armyworm, *Spodoptera exigua* [38], while it reduced survival, biomass and caused longer instar durations in *Lacanobia oleracea* [12, 62]. In an earlier study, ConA was reported to cause 90% mortality and retarded development on the lepidopteran pest *L. oleracea*. However, the study also revealed that ConA had no significant effect on the larval weight [15]. The insecticidal property of ASAL from *A. sativum* and APA from *A. porrum* was reported in transgenic tobacco by Sadeghi et al. [44, 45]. Both lectins caused decreased weight, delayed development, increased mortality and impaired pupation in *Spodoptera littoralis.* Further, the lectin from *Dioscorea batatas* (DB1) affected the metamorphosis from pupae to larvae of *Helicoverpa armigera* [39]. The results from the present study is similar to the earlier reports, wherein WsMBP1 caused increased mortality, reduced weight gain and delayed metamorphosis from larval to pupal stage in *H. puera*.

The insecticidal effect of several mannose-binding lectins is reported against hemipteran pests. GNA was reported to significantly affect the survival of *Nephotettix virescens* and *N. lugens* nymphs [14], *Sogatella furcifera* [37] and *Laodelphax striatellus* [63] in transgenic rice. The lectin caused decreased feeding, reduced fecundity and delayed development. Similarly, ectopic expression of ASAL in chickpea affected the survivability and fecundity of phloem feeding *Aphis craccivora* [8]. Con A is also reported to have detrimental effect on sucking insects like *Rhopalosiphum*

padi [53] and *Acyrthosiphon pisum* [48]. The major effect of ConA against insects included increased mortality, decreased fecundity and delayed development. The detrimental effect of ASAL on survival and development of sucking insect pests is reported against white backed plant hopper, brown plant hopper and green leaf hopper [64]; *Lipaphis erysimi* [11]; red cotton bug [5], mustard aphid [42]; green leaf hopper [30] and *Phenococcus solenopsis* [1]. In another study, ectopic expression of *A. cepa* agglutinin (ACA) in transgenic mustard was reported to be toxic against mustard aphid when compared to ASAL and GNA [18].

The salt-inducible lectin Orysata from rice was reported to have strong insecticidal activity against *Spodoptera exigua, Myzus persicae* and *Acyrthosiphon pisum* in transgenic tobacco. It caused increased mortality, reduced weight and extended larval development phase [2]. In a recent report by Javaid et al. [21], expression of Blue Mountains funnel-web spider neurotoxin (Hvt) and onion leaf lectin in tobacco showed 100% mortality in three hemipteran pests, *Phenacoccus solenopsis*, *Myzus persicae* and *Bemisia tabaci*. Similarly, the detrimental effect of WsMBP1 against the hemipteran pest *P. sanguinolens* was recorded in the present study, causing significant mortality and severely impaired molting as documented in other mannose-binding lectins.

In spite of numerous reports on insecticidal property of lectins, the exact mode of action of lectins in providing resistance against insects remains unclear. In the present study, histological analysis of lectin-treated midgut revealed disrupted and diffused secretory cells surrounding the gut lumen in larvae of *H. puera* and *P. sanguinolens.* The damage noticed in the gut of both insects suggests the interference of WsMBP1 with the secretory layer of the gut lumen, thereby revealing that this lectin probably affected the insect secretory mechanism and hampered food uptake in larvae of the tested insect pests.

Lectins have been reported as a prospective candidate for imparting tolerance against different classes of insect pests and specifically against sap-sucking insects. The effects of *W. somnifera* lectin against different groups of insect pests suggest its potential as an insecticidal gene for transgenic research toward generating insect pest-tolerant plant genotypes.

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