A Highly Abundant Lectin Protein in *Arabidopsis thaliana* Confers Resistance Against Pathogens

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ABSTRACT

Lectins are glycoproteins that recognize and bind to specific carbohydrates. They are involved in a range of biological functions, such as plant defence, storage proteins seed germination and plant microbe interactions. Lectin 3.1 (At3g15356) is a protein in plant model, *Arabidopsis thaliana*, that has been shown to be up-regulated in all defence pathways, especially in response to methyl ester jasmonate (MJ). All that was known about the gene was that it had good homology to the beta domain of legume lectins. That aim of this project was to characterize the structure and function of the lectin protein using CD spectra and X-ray crystallography. A T-DNA insertion line for the lectin gene and a number of 35S over-expression lines that had varying levels of expression had been generated, but none of these showed any obvious phenotype. Two protein bands were observed on Coomassie stained SDS-PAGE gels in the over-expression lines and in MJ induced wild-type (WT). The two protein bands represented two isoforms of the lectin 3.1 protein; in a glycosylation assay the larger protein band was shown to be heavily glycosylated. A nematode (*M. incognita*) disease assay discovered that the lectin over-expression lines had less nematode eggs compared to that of the WT and that the insertion line had more nematode eggs than the WT. This data provides evidence that lectin 3.1 improves plant resistance against *M. incognita* infection. Interestingly, the nematode gut lining contains fucose with which lectin 3.1 binds to.

Keywords: *Arabidopsis thaliana*, lectin's structure and function, pathogen resistance

INTRODUCTION

Lectins, glycoproteins that bind to sugar residues reversibly and specifically, found in animals, plants and microorganisms (Hartmann *et al.*, 2006). Recent studies revealed that lectins in plants are essential for protein-protein interactions via lectin receptor kinases and RGD ligands (Gouget *et al.*, 2006). In addition, the mannose-specific intracellular lectin located in the ER-Golgi and mannose-phosphate receptors of the P-type lectin family which bind to oligosaccharide ligands, release in the acidic pH of the lysosome (Sharon & Lis, 2004).

A recent study reported that lectins might function as anti-nematode proteins (Burrows *et al.*, 1998). Experiments have been conducted on nematodes such as the study on the galling of the tomato roots by *M. incognita* and reducing the disease by soil application of Concanavalin A (Con A) or *Limax flavus* agglutinin (Marban-Mendoza *et al.*, 1987). For the past years, details of the role of lectin in resistance against *M. incognita* were limited. Studies have been done previously on model plants and it was useful as it interacts with a few plant nematodes species (Sijmons, 1991; Urwin, 1997).

Lectin structures from over 80 species have been characterized to solve the secondary structure and tertiary structure in order to determine lectin biological functions. Many studies revealed the structure through circular dichroism (cd) spectrum of lectin indicating 10% á-helix, 38% â-sheet, 28% unordered form and

6% of proline conformation (de Oliveira *et al.*, 2003). Crystal tertiary structures from *Viscum album* exhibited hexagonal shape of lectin at 1.9 ú that binds to adenine mimicking RNA substrate binding (Krauspenhaar *et al.*, 2002) and at 2.8 ú showing RNA-glycosidase activity and binds to galactose specifically (Krauspenhaar *et al.*, 1999).

However, the structure and functions of lectins from *A. thaliana* are not well characterized. Therefore, this experiment was conducted to solve the protein structure and function of lectin-like protein in *A. thaliana*. The functional studies compared the performance lectin protein from *A. thaliana* that bind to various types of sugar residues and that had either an increase or decrease in gene expression of lectin, with a wild type control, against *M. incognita*.

Materials and Methods

RNA extraction from *Arabidopsis thaliana*

Frozen scions were used and ground in liquid nitrogen to extract RNA from the plants. A protocol from Promega called SV Total RNA Isolation System was used, as per the manufacturer's instructions. The harvested RNA was used for PCR to amplify the DNA.

Plant transformation

PCR products were subjected to pKEN containing the HindIII and EcoR1-digested products and the D35S promoter (see manufacturer's protocol) yielding entry clones. The resulting construct were transformed in *Agrobacterium tumefaciens* using electroporation method and grown in LB media. Then *A. thaliana* flowers were dipped in the media containing *A. tumefaciens*. The flowers containing the cloned seeds (lectin overexpression) with antibiotic resistance genes were grown for 5 weeks.

Large scale growing of *Arabidopsis* with and without MJ induction

Arabidopsis plants were grown in CSIRO lab under a day/night regime of 8/16 h, respectively. After 5 weeks, the plants without MJ induction were harvested for extraction and purification. The plants with JAME or MJ (jasmonite methyl ester) induction were induced 5 days before harvesting time (Chen *et al.*, 2002).

SDS/PAGE

Native gel electrophoresis using 12% polyacrylamide gels was carried out in alkaline buffer system or precast gel electrophoresis using 4-12% NuPage gels (Invitrogen) were also used to determine the molecular size of the protein.

Circular dichroism

The c.d. readings were taken on a Jasco J-710 recording spectropolarimeter under constant nitrogen flush. CD spectra were analyzed using K2D program and compared using Psipred program and DSSP program.

Crystallization screening

Crystal screening of lectin was carried out using the hanging drop vapor-diffusion method (Hampton Index I, Hampton Index II, Emerald Wizard I, Emerald Wizard II and Emerald Biosystems) at 4°C and room temperature (to determine protein concentration and the crystal conditions of precipitant). The crystal screening and optimization (Mishra *et al.*, 2004; 2005) were carried out for 1-2 weeks before x-ray intensity data collection done at X-ray crystallography unit at Institute of Molecular Bioscience, UQ.

Functional Assay

Harvesting nematode eggs

The nematode eggs that were used in this study were from *Meloidogyne incognita*. The eggs were harvested from tomato plants at the Department of Primary Industries and Fisheries (DPI and F). The eggs were then kept at 10-11°C before inoculation into the *Arabidopsis* root zone. The number of *Arabidopsis* plants used was 90, consisting of 3 repetitions of 3 treatments with 10 plants each. The treatments were a wild type control, 35S lectin over expressing transgenic lines, and lectin insertion lines.

Infection of Arabidopsis

Nematode eggs were transferred into the soil close to the root system using a pipette. The numbers of eggs were chosen to provide a good infection rate in numbers occuring in normal circumstances; only half the number of the eggs hatched. The *Arabidopsis* that were used were two weeks old.

Growth conditions of infected Arabidopsis

The *Arabidopsis* were grown on University of California potting mix (UC mix) an the light condition of the incubator were 8 h of light from 10:00 am to 18:00 pm daily.

Nematode eggs harvest after one life cycle

The eggs were harvest after 6 weeks of infection. The roots were washed in running water to eliminate of growth medium and followed by a 1% sodium hypochlorite (NaClO) treatment.

Nematode egg counting

The nematode eggs were counted under a light microscope on a 20 line counting slide. The samples were re-filtered twice to remove excess organic materials.

RNA extraction

Frozen scions were used and ground in liquid nitrogen to extract RNA from the plants. A protocol from Promega called SV Total RNA Isolation System was used, as per the manufacturer's instructions. The harvested RNA was used for real time PCR.

RNA isolation and cDNA synthesis

One biological replicate of total RNA was isolated from 100 mg of *Arabidopsis* foliar tissue ground up as a large combined prep in liquid nitrogen and extracted using the Promega™ SV RNA isolation kit.

Real-Time Quantitative PCR conditions and analysis

RT-Q-PCR of the cDNA samples was performed at the School of Molecular and Microbial Siences, The University of Queensland. Analysis was carried out in ABI optical 384-well plates using an ABI PRISM© 7900 HT Sequence detection System (Applied Biosystems).

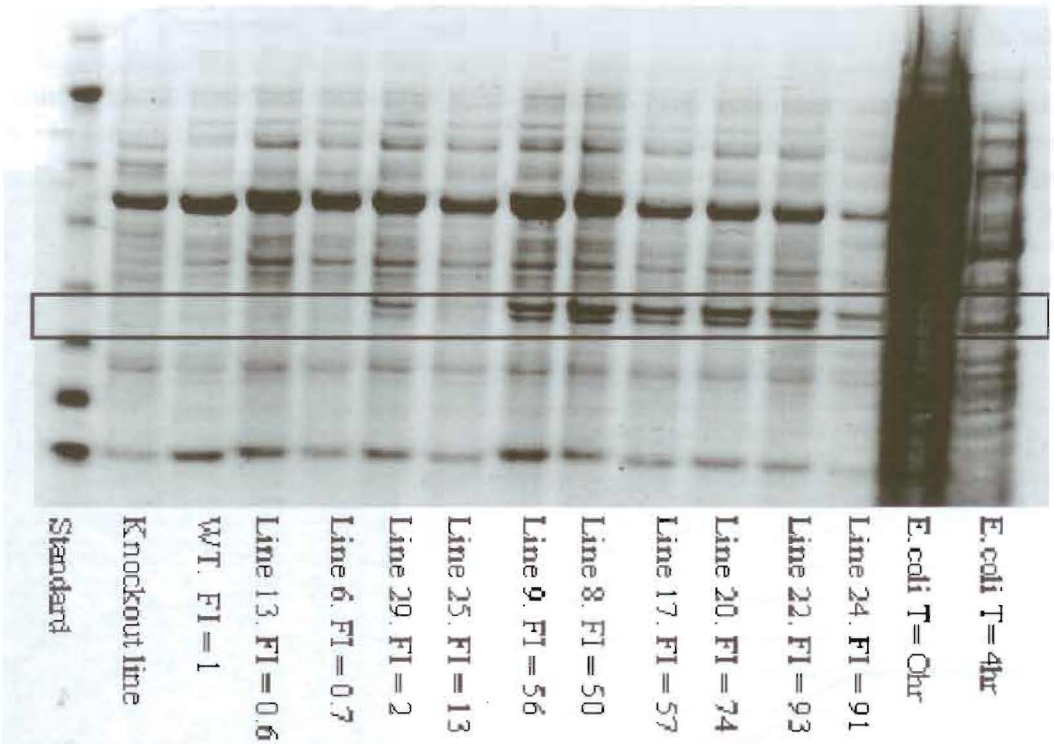

Figure 1. SDS page stained with Coomassie blue showed the double band in lectin that was precipitated with ammonium acetate

Results and Discussion

Protein overexpression from *A. thaliana*, as described in legume lectin family protein, is presented below. In Fig 1, the double-band of the lectin proteins was heavily glycosylated appearing in the overexpressing plants (lines 17, 20, 22, 24, 29) and in jasmonate-treated *Arabidopsis* plants (lines 8 and 9). In *E. coli* (lines *E. coli* T), the non-glycosylated form was found in inclusion bodies (not in the soluble fraction). Line means individual over-expressing 35S transformant and FI means fold induction (expression of 35S transformant divided by expression of wild type). In addition to Fig 1, SDS/PAGE stained with Coomassie blue showed the double band in lectin precipitated with ammonium acetate. This double-band of the lectin proteins was heavily glycosylated appearing in the overexpressing plants and in jasmonate-treated *Arabidopsis* plants. While in *E. coli*, the non-glycosylated form was found in inclusion bodies (not in the soluble fraction).

Circular dichroism

Turning into CD spectra experiments to characterize secondary structure of this protein, we observed that lectin from *Arabidopsis* plants without MJ induction (Fig 2) showed correct folding protein with CD values of 6% á-helix, 44% â-sheet and 50% random coil that values were in the range of PsiPred and DSSP structure prediction (5.5-7% á-helix, 43-44% â-sheet and 49-51.5% random coil).

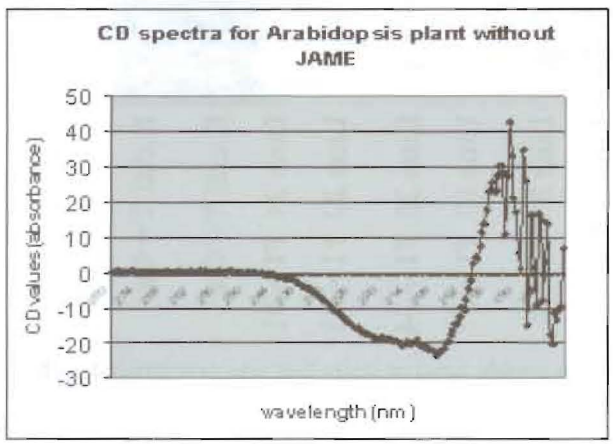

Figure 2. CD spectra of lectin protein from *Arabidopsis* plant without JAME (MJ) induction showed 6% of á-helix, 44% of â-sheet and 50% of random coil

Crystallisation

In Fig 3, each crystal was at least 1 mm long and the size and shape of polyhedral crystals from batch to batch, one of them is *Arabidopsis* crystal with no diffraction and others are salt crystals. Moreover, as can be shown in Fig 3, *Arabidopsis* lectin crystals that were grown for 1 week at cold room (4°C) by the vapor diffusion technique, had at least 1 mm long of polyhedral shape of crystals. One of the crystals was *Arabidopsis* lectin crystal with no diffraction and could not be visualized in X-ray diffraction because it was neither salt nor protein, presumably due to high abundance of sugar residues attached to the glycosylation sites of protein, while the other crystals were salt. Therefore, it is important for future experiments to have pure protein using carbohydrate affinity chromatography, PGNase enzyme to cleave the sugar residues that attached to N-glycoslation sites of the protein and optimize the best conditions of precipitants to grow lectin crystals as well as rapid detection to identify between salt crystal and protein crystal (such as IZIT crystal dye from Hampton), prior to X-ray crystallography or use another approach using NMR which is better to determine protein structure with high pI (lectin has pI = 8.00).

Figure 3. *Arabidopsis* lectin crystals grown for 1 week at cold room (4°C) by the vapor diffusion technique

Tertiary structure prediction

Taken into together, we performed structure prediction, as shown in Fig 4. tertiary structure prediction obtained from Swiss model and DSSP showed 7 major β-strands (7% of β-sheet) and 44% of α-helix and 49% of random coil. Moreover, we found that tertiary structure prediction viewed by Swiss model and WebLab Viewer Lite showed surface charge of lectin protein (blue areas for positive charge, red areas for negative charge). These positive charges bound to cation exchange chromatography (negative charge MonoS column). These positive charge surfaces were the active sites that bound to cation exchange chromatography. Furthermore, the binding pockets that was in the middle of the structure where β-sheets located may recognize sugar residues specifically at the configuration of hydroxyl at single carbon atoms (C-2 and/or C-4).

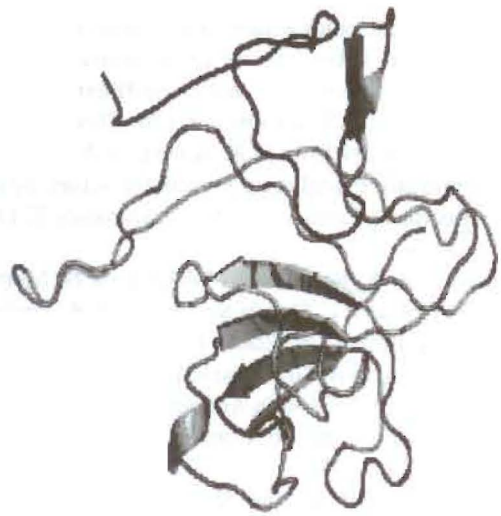

Figure 4. Tertiary structure prediction showed from Swiss model and DSSP showing 7 major β-strands (7% of β-sheet) and 44% of α-helix and 49% of random coil

Functional assay of nematocidal effects in Arabidopsis plant overexpressing lectin

The data in Figs. 5, 6 and 7 showed that the wild type *Arabidopsis* had the least resistance and lectin insertion line had expressed less resistance against *M. incognita* and 35S lectin overexpression line had the most resistance against *M. incognita*. Moreover, with lectin 2 and 4, the lectin insertion had a higher relative abundance to wild type compared to 35S.

This might be due to the compensation of the lectin expression on lectin 3.1, while lectin insertion showed little difference in the relative abundance to wild type. These studies concluded that the lectin protein does have the potential to inhibit nematode infection from Fig. 5 because the P value was not significant compared to the other two P values (Figs. 6 and 7), might be due to the samples size. There were some missing values from the data. Therefore that contributes to the error (shown on the error bar). Taken into account, the data showed that lectin provided resistance against *M. incognita*, therefore, future experiment should be done by designing stronger 35S construct to reduce the infection, using sandy mix to remove more of the peat moss that would improve the accuracy of the egg counts and providing the nematode's choice of plants. In Fig 8, lectin 3.1 with 35S was relative abundance of lectin DNA expression to wild type, that is, 57 times higher that lectin insertion.

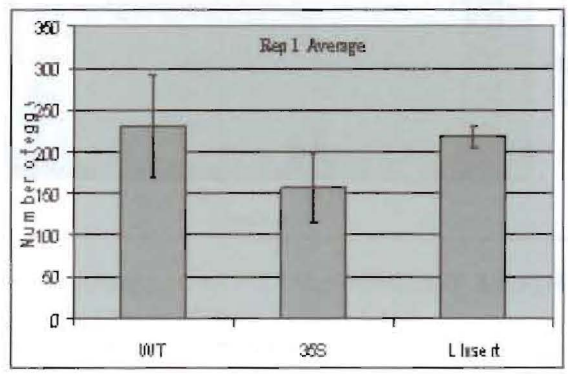

Figure 5. Comparison between the average numbers of eggs produced by *M. incognita* on *Arabidopsis* wild type, 35S and insertion line in Rep 1 (P=0.3654)

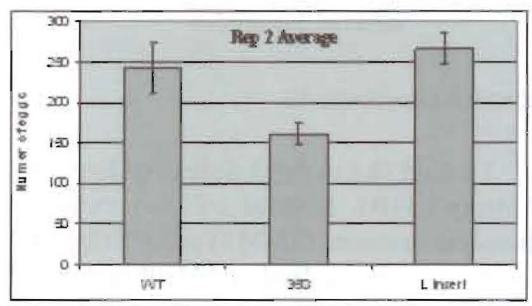

Figure 6. The data in Rep 2 (P=0.0064) showed that the lectin insertion line had the least resistance as well as wild type *Arabidopsis* and 35S lectin overexpression line had expressed the most resistance against *M. incognita*

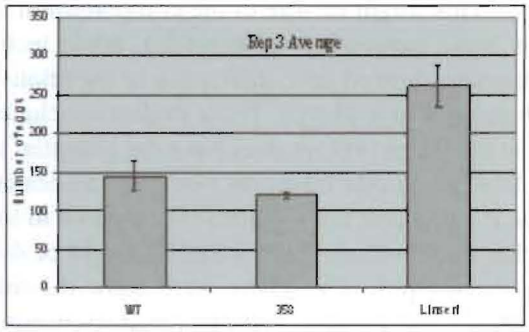

Figure 7. The data in Rep 3 ($P<0.0001$) showed that the lectin insertion line had the least resistance and 35S had expressed resistance against *M. incognita* and wild type *Arabidopsis* had also resistance against *M. incognita*

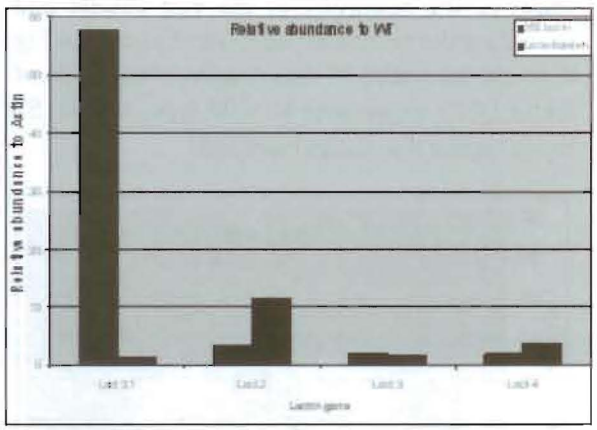

Figure 8. The data revealed 35S lectin overexpression line 3.1 had 57 times the amount of lectin expression compared to lectin insertion (relative abundance measured by RT-PCR). While lectin lines 2 and 4, and the lectin insertion had a higher relative abundance to wild type compared to 35S. Here, we intended to investigate the functional study of lectin in plant infected nematodes

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References

Burrows PR, Barker AD, Newell CA, WDO & Hamilton (1998) Plant-Derived Enzyme Inhibitors and Lectins for Resistance Against Plant-Parasitic Nematodes in Transgenic Crops. Pestic Sci 52176-52183.

Annales Bogorienses n.s. vol. 12. no. 1, 2008

Chen Y, Pneumans WJ, Hause B, Bras J, Kumar M, Proost P, Barre A, Rouge P & Van Damme ELM (2002) Jasmonate Methyl Ester Induces the Synthesis of a Cytoplasmic/Nuclear Chitooligosaccharide-Binding Lectin in Tobacco Leaves. The FASEB J 10.1096/fj.01-0598fje.

De Oliveira ML, Beltramini LM, de Simone SG, Brumano MHN, Silva-Lucca RA, Nakaema MKK, Pires CV & de Almeida Oliviera MG (2003) Purification and Partial Characterization of a Lectin from *Caesalpinia tinctoria* Domb, Ex Dc Fruits Braz J Plant Physiol 15 (2): 119-122.

Gouget A, Senchou V, Govers F, Sanson A, Barre A, Rouge P, Pont-Lezica R & Canut H (2006) Lectin Receptor Kinases Participate in Protein-Protein Interactions to Mediate Plasma Membrane-Cell Wall Adhesions in Arabidopsis Plant Physiol 140: 81-90.

Hartmann M, Nikita P & Keusgen M (2006) Innovative Analytical System for Screening on Lectins. Biosensors and Bioelectronics 22 (1): 28-34.

Krauspenhaar R, Rypniewski W, Kalkura N, Moore K, DeLucas L, Stoeva St, Mikhailov A, Voelter W & Betzel Ch (2002) Crystallisation under Microgravity of Mistletoe Lectin I from Viscum Album with Adenine Monophosphate and the Crystal Structure at 1.9 ú Resolution. Acta Crys D58: 1704-1707.

Krauspenhaar R, Eschenburg S, Perbandt M, Kornilov V, Konareva N, Mikailova I, Stoeva S, Wacker R, Maier T, Singh T, Mikhailov A, Voelter W & Betzel C (1999) Biochem Biophysic Res Comm 257: 418-424.

Marban-Mendoza N, Jeyaprakash A, Jansson HB & Zuckerman BM (1987) Control of Root-Knot Nematodes Tomato by Lectins. J Nematology 19: 331-335.

Mishra V, Bilgrami S, Sharma RS, Kaur P, Yadav S, Krauspenhaar R, Betzel C, Voelter W, Babu CR & Singh TP (2005) Crystal Structure of Himalayan Mistletoe Ribosome-Inactivating Protein Reveals the Presence of a Natural Inhibitor and a New Functionally Active Sugar Binding Site. J Biol Chem 280 (21): 20712-20721.

Mishra V, Ethayathulla AS, Sharma RS, Yadav S, Krauspenhaar R, Betzel C, Babu CR & Singh TP (2004) Acta Crystallography D60: 2295-2304.

Sharon N & Lis H (2004) History of Lectins: from Hemagglutinins to Biological Recognition Molecules. Glycobiology 14 (11): 53R-62R.

Sijmons PC, Grundler FMW, Von Mende N, Burrows PR & Wyss U (1991) *Arabidopsis thaliana* as a New Model Host for Plantparasitic Nematodes. Plant J 1: 245-254.

Urwin PE, Lilley CJ, McPherson MJ & Atkinson HJ (1997) Resistance to Both Cyst and Root-Knot Nematodes Conferred by Transgenic *Arabidopsis* Expressing a Modified Plant Cystatin Plant J 12: 455-461.