Research Article

Isolation and characterization of mannose-binding lectin gene from leaves of Allium ascalonicum (Shallot) and its putative role in insect resistance

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Abstract

Plant lectins are the heterogenous group of glycoproteins extensively studied for their potent insecticidal property against Hemipteran pests. In this present study, the full-length cDNA of monocot mannose-binding insecticidal lectin gene was isolated from Allium ascalonicum leaves. The isolated Allium ascalonicum Lectin (AAL) gene was cloned in pGEM-T vector, sequenced and the sequence was submitted to GenBank (KM096570.1). Sequence analysis revealed a 468 bp open reading frame (ORF) encoding a putative 155 amino acids agglutinin precursor. Multiple sequence alignment and phylogenetic analysis of AAL amino acid with those of 30 other Mannose binding lectin (MBL) sequences in NCBI revealed a high similarity of 85-95% indicating that AAL is a member of the MBL super family and forms a cluster with other onion lectins. Secondary structure prediction and the homology modeling showed that AAL protein possess predominantly β-sheets and three potential mannose-binding motifs consisting of 5 amino acid residues QDNVY like other GNA lectins. The results of the in silico analysis predict that the Allium ascalonicum lectin gene can be another potent insecticidal protein.

Keywords: Hemipterans, Allium ascalonicum, Mannose binding lectins, In-silico analysis

1. Introduction

Increasing the yield of economically important crops by crop protection is a very important issue in modern agriculture. It has been estimated that yield loss in crop plants due to insect pests is around 13-14% worldwide (Gatehouse et al., 1998). Chemical pesticides are widely used for protection against insects but it leads to several undesirable effects on human and environment. Hence, genetically engineering the insecticidal gene into the crops is the preferred choice for protection against pests. In past few years, several genes with insecticidal property have been identified which includes lectins, ribosome-inactivating proteins, protease inhibitors, α-amylase inhibitors, arcelin, canatoxin-like protein, ureases and chitinases (Carlini et al., 2002). All the
above mentioned proteinaceous inhibitors are naturally occurring defense mechanisms in plants to cope with the continuous threat from different phytophagous insects. The potential for using the natural plant genetic barriers has increased with the development of gene transfer techniques. Among several plant proteins, lectins are well documented for their potential effects against the insect pests and for transgenic based pest control strategies (Rao et al., 1998; Saha et al., 2006; Vandenborre et al., 2011; Macedo et al., 2015).

Lectins are heterogenous group of proteins of non-immune origin possessing at least one non-catalytic domain that specifically binds to carbohydrates. The physiological role of these lectins in plants is widely debated ranging from growth regulation (Howard et al., 1972), plant development (Brill et al., 2001), seed storage (Huang et al., 2006), and defense against pest and pathogen (Macedo et al., 2003; Luo et al., 2007). Among different lectins, lectins related to snowdrop lectin Galanthus nivalis agglutinin (GNA) have been well studied for its insecticidal property and several novel GNA like lectin genes were identified and characterized from different species of Alliaceae (Bandyopadhyay et al., 2001; Dutta et al., 2005; Chandrasekhar et al., 2014) and Araceae (Majumder et al., 2005). One major reason for large interest in GNA-related lectins is that, several of these lectins are found in edible plants like garlic, onion, etc which will reduce the problems related to consumer acceptability whenever these lectins would be used in crop plants. The harmful effects of lectins on insects are larval weight decrease, mortality, feeding inhibition, delay in larvae total developmental duration and adult emergence, fecundity on the first and second generation (Powell et al., 1993).

In this paper, we have reported the isolation, cloning and characterization of mannose binding lectin gene from Allium ascalonicum (Shallot) leaves. The deduced amino acid sequence of the AAL gene was used to ascertain the evolutionary position of the isolated gene in the Alliaceae family. In addition, the secondary structure and the three dimensional structure of the AAL protein was also predicted.

2. Materials and methods
2.1. Isolation of AAL gene
The fresh onion leaves were collected from a farmland in Madurai district, Tamil Nadu, India. The total RNA was extracted from the onion leaves using liquid nitrogen by TRIzol method. The isolated total RNA was used for first strand cDNA synthesis using oligo-dT primer and reverse transcriptase provided with the Monster Script cDNA synthesis kit (Epicentre Biotechnologies, Madison, WI). The AAL gene was amplified using the forward primer 3'-AAGCTTTGCTGGCCAGAACG TATTGG -5' and the reverse primer 3'-GAGCTCTCCAAATTCAGCGACGCAGCTG- 5' with Hind III & Sac I restriction sites respectively. The general PCR program was preceded by a 5 min denaturation (94°C). Subsequently for 30 cycles, each cycle by 1 min denaturation (94°C) followed by a 1 min annealing period at 55°C - 65°C & extension at 72°C for 1 min, the final extension at 72°C for 7 min. The PCR fragment was cloned in pGEM- T (Promega) vector and sequenced.

2.2. In-silico analysis of Allium lectins
All Amino acid sequences used in this study were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). A total of 31 sequences of MBL’s from Alliaceae family including the isolated AAL gene were collected from NCBI. Sequences with significant identity (85-95%) were aligned with ClustalW algorithm implemented in Molecular Evolutionary Genetic Analysis (MEGA 6) (http://www.megasoftware.net) by using distance matrix. Neighbor Joining (NJ) trees were constructed with 1000 bootstraps at uniform divergence rates with distance 'p' as the evolutionary model (Tamura et al., 2013). The secondary structure of the deduced amino
acid was predicted using PSI-PRED (http://bioinf.cs.ucl.ac.uk/psipred/) online server (McGuffin et al., 2000).

2.3. Homology modeling of AAL
Homology model of AAL was constructed by comparative modeling in SWISS-MODEL (http://swissmodel.expasy.org). SWISS-MODEL is a fully automated protein structure homology modeling server, accessible via ExPASy web server. The model was visualized using Pymol Viewer (DeLano 2002; Schrodinger 2010). The model was then energy minimized and refined using modrefiner refinement tool (http://zhanglab.ccmb.med.umich.edu/ModRefiner/) (Xu et al., 2011). The model was validated by Verify 3D analysis from SAVES server (http://services.mbi.ucla.edu/SAVES/) which determines the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment (alpha, beta, loop, polar, nonpolar, etc) and comparing the results to good structures (Bowie et al., 1991; Lüthy et al., 1992). In addition, PROSA (Wiederstein and Sippl 2007) which calculates an overall quality score for a specific input structure and PROCHECK (Laskowski et al., 1993) which checks the stereo chemical quality of a protein structure by analyzing residue-by-residue geometry and overall structure geometry by Ramachandran plot analysis were used.

3. Results
3.1. Isolation and cloning of AAL
The total RNA was isolated from onion leaves by Trizol method and using oligoDT primers & reverse transcriptase mRNA was converted to cDNA. This cDNA was used to amplify the 508 bp AAL gene using gene specific primers with Hind III and Sac I enzymes in forward primer and reverse primer respectively (Fig. 1). The primers were designed from the AAL sequence available in the NCBI database (GenBank: L12172.1). The amplified fragment was cloned in pGEM-T vector and the clone pRI1 was confirmed by multiple enzyme digestion (Fig. 2A and Fig. 2B). The clone was sequenced (Fig. 3). The sequenced AAL gene had an ORF of 468 bp encoding a 155 amino acid protein.

Fig. 1. PCR amplification of lectin gene from cDNA of onion.
Lane 1- 1kb DNA ladder, Lanes 2-7 – different cDNA concentrations used for amplification (cDNA was diluted to 1:4 &1:8 ratio and different volumes of cDNA 1 µL, 2 µL, 3 µL from each was used for PCR amplification). Lane 8 – 100 bp DNA ladder.
Fig. 2A. Plasmid map of pRI1 clone.
pRI1 is a 508 bp amplicon of lectin gene from *Allium ascalonicum* with Hind III & Sac I enzyme sites cloned in pGEMT vector of size 3 kb. Total size of the clone 3508 bp.

Fig. 2B. Multiple enzyme digestion of the clone; Lane 1-1kb DNA ladder, Lane 2,3,4 – Digestion of pRI1 plasmid with enzymes Hind III, Pst I and Sac I respectively.

![Fig. 2B](image)

3.2. Sequence Analysis of AAL gene
The AAL gene had 93-95% similarity with the mannose binding lectins of other Allium varieties. From the deduced amino acid sequence, three mannose binding domains, with the conserved amino acids QDNVY were identified, which authenticates its insecticidal property (Fig. 4).

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ATG GCCGCAAAACGTATTTGCGCAAAACGAAGACTGTACGAGGAACAGTACAGTTTTATAGATGTCTGGATCTTTTATATGCAGGATGACTGCAACCTTGTACTGTACGAATACAGCACCCCAATCTGGGCCTCAAACACGGGCGTCACCGGCCAAAGAACGGGTGCAGGGCCGTGATGCAGGTTGACGGCAACTTTGTCGTCTACGATGTTAAGGGGCGTGGCCGTCTGGGCCAGTAACAGCAGAAGAGGGAACGGAAACTACATCCTGGTGCTTCAGAAGGACAGGAACGTTGTATTTATGGATCTGATATTTGGTCTACTGGTACGTACAGGAAAAAAGAGGGTGGAGCAGTTGTTATGGCAATGAGATGGTACTGTTGATGGGGGCTCCGTGATTGGACCGGTAACGGTGAATCAGAACGTCACTGCCGTCCGAAAAGAAAGCAGCTACTGCTGCTGCTGCTTGAATTTAGAGATGAGTTCTATGTTTTGAGTATGAACAATAAA
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Fig. 4. Deduced amino acid sequence with 3 mannose binding motifs consisting of conserved amino acids QDNVY; Mannose represented as boxes in green, 3 mannose binding motif amino acids in pink, green & red respectively.

3.3. Phylogenetic analysis of AAL sequence
Multiple sequence alignment of different *Allium* lectins was performed using ClustalW of MEGA 6 tool. The phylogenetic neighbor-joining tree was constructed with 31 *Allium* lectins including the AAL using MEGA 6 tool (Fig. 5). The complement of the probability (1 - α) is computed in MEGA 6 and it is called as confidence probability (CP). The reliability of a branch length is high when the CP is high, thus the branch length is considered to be statistically significant. MEGA 6 inferred the evolutionary tree by a Neighbor-Joining (NJ) algorithm by using a matrix of pairwise distances. Bootstrap confidence levels are shown as percentages on nodes and confidence values are shown in branches. The constructed phylogenetic tree showed that isolated AAL belongs to the MMBL super family and sub clustered with other onion lectins.

Fig. 5. Phylogenetic neighbor-joining tree of 31 *Allium* lectin gene sequences showing the relationship between AAL and other *Allium* lectins. The isolated AAL gene is highlighted in brown box. Bootstrap confidence levels are shown as percentages on nodes and confidence values are shown in branches.
3.4. Secondary structure prediction of AAL

The secondary structure of AAL was predicted using PSI-PRED online tool (Fig. 6). The subunits of MBL should assemble into a stable dimer by exchanging their C terminal β-strands to form a hybrid β-sheet, which is crucial for its insecticidal activity (Mondal et al., 2011). The predicted secondary structure of AAL assembles in to a stable hybrid β-sheet thus confirming its potent insecticidal property.

Fig. 6. Secondary structure of AAL predicted using PSI-PRED online tool. Secondary structure of AAL showing β-strand (yellow) and coil (Black line) at the corresponding amino acid sequence.

3.5. Homology modeling of AAL protein

The knowledge of 3D structure of a protein provides an insight into its structure-function relationship. Among different templates aligned, template with PDB ID: 1kj1D showed 74.3% of identity to the AAL protein sequence which was used for automated comparative modeling using SWISS-MODEL. The generated 3D structure of AAL and the binding pockets present were shown in figure 7. The model was further energy minimized and refined using Modrefiner refinement tool.

Fig. 7A. 3D structure of AAL model with mannose binding sites predicted using SWISS-MODEL using the template of PDB ID: 1kj1D.
3.6. Validation of the AAL model

The generated homology model was validated using PROCHECK, VERIFY 3D and PROSA. According to the Ramachandran plot analysis using PROCHECK, a good quality model would be expected to have more than 90% of residues in the most favorable region. Ramachandran plot of model revealed that the phi-psi torsion angles for 91.6% of residues are in the most favorable region and 3.2% in additionally allowed region. Thus the structural analysis confirmed that the obtained model was stereo chemically good (Fig. 8).

![Ramachandran Plot](image)

**Fig. 8.** Ramachandran plot analysis of AAL model analyzed using PROCHECK. The most favorable conformation of $\Phi - \Psi$ values are colored red, additional allowed, generously allowed and disallowed regions are indicated as yellow, light yellow and white fields, respectively.

The model was then analyzed using Verify 3D analysis in SAVES server. 100.00% of the residues had an averaged 3D-1D score $\geq 0.2$. Thus, confirming that the quality of the predicted AAL structure is comparable to the already available good structures (Fig. 9). The model was then validated using PROSA. It predicts the z-score of the model, which indicates the overall model quality. It can be used to check whether the z-score of the input structure is within the range of scores typically found for native proteins of similar size. The z-score of AAL was predicted to be -6.44 thus confirming that the predicted AAL structure falls within the range allowed for experimentally determined structures (Fig. 10).

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Fig. 9. Verify 3D profile of predicted AAL model. The Y-axis represents 3D–1D Averaged score and X-axis represents the residue number of the protein.

Fig. 10. PROSA analysis of AAL model. Z-score of predicted AAL model. X-axis indicates the number of residues and Y-axis indicates Z-score. Black dot represents the range of Z-score of the AAL within the native conformation of crystal structures, dark blue-colored region represents the conformation of structure determined by Nuclear Magnetic Resonance (NMR) and light blue-colored region represents the conformation of structure determined by X-ray crystallography.

4. Discussion

Use of novel genes through genetically modified plants to increase the level and range of resistance to insect pests is one of the potential strategies. Considerable progress has been made in developing transgenic plants with various genes from Bacillus thuringiensis in different crops (Sharma et al., 2007). However, there are distinct possibilities of development of resistance to these genes (Tabashnik 1994) and hence there is need to identify alternative genes such as lectins for deployment through transgenic crops. Lectins are known to have more of chronic than acute effect on insects (Vandenborre et al., 2011). The lectins from Allium sativum had been successfully used to confer insect resistance in tobacco, Brassica and rice (Dutta et al., 2005, Bharathi et al., 2011, Bala et al., 2013). In this study, we have isolated lectin gene from Allium ascalonicum. The phylogenetic analysis of the deduced amino acid sequence indicated that AAL protein belongs to the MMBL super family and sub-clusters with other onion lectins. The secondary structure prediction of AAL revealed that the protein possess predominantly β-sheets and possess 3 mannose binding motifs of repeat sequences (QXDXNXVXY) which plays a major role in alpha D-mannose recognition (Hester et al., 1995; Ramachandraiah and Chandra 2000; Mondal et al., 2011). The 3-Dimensional structure of AAL was predicted using Swiss modeller and was validated using PROSA, Verify 3D and Ramachandran plot analysis of PROCHECK. The
generated model was 74.3% identical to the X-ray crystallographic structure of *Allium sativum* lectin (PDB ID: 1kj1D). The generated model can further be used for analyzing the docking of AAL to the insect glycoprotein receptors. The in-silico analysis provides an insight into the fact that the Allium ascalonicum can be a potent insecticidal protein like other insecticidal *Allium* lectins identified so far.

5. Conclusion

From the present study, we conclude that the lectin gene isolated from the leaf of *Allium ascalonicum* can be safely used in a heterologous system for developing insect resistance against Hemipteran pests.

Conflict of interest statement

We declare that we have no conflict of interest.

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References


