International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 7(1): 1-10 March 2014
DOI 10.5958/j.2230-732X.7.1.001

Biotechnology

Cloning and Molecular Characterization of *LECASAI* Lectin Gene from Garlic (*Allium Sativum* L.)

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Paper No. 172 Received: October 12, 2013 Accepted: January 14, 2014 Published: March 07, 2014

Abstract

Lectins have always demonstrated their role in bearing insecticidal activity. They are although considered to be toxic for mammals but some studies have reported that the presence of mannose binding region is believed to impart non-toxic nature to lectins against mammals including humans. In the present investigation, the research was conducted to elucidate the relationship of lectin gene *LECASAI* with other previously cloned lectins having insecticidal activity and to ensure the presence of the conserved mannose binding region/site in *LECASAI*. In this study, we report isolation of *LECASAI via* PCR, Cloning, Characterization & Bioinformatics analysis of *LECASAI* isolated from Garlic (*Allium sativum* L.). The full length gene sequence of *LECASAI* obtained from sequencing consisted of 1029bp which was subjected to *In-silico* analysis in order to determine its ancestral relationship and the presence of the conserved mannose binding region within the sequence. Results from *In-vitro* analysis indicated a higher homology of *LECASAI* with those of insecticidal lectins and the presence of mannose binding region in *LECASAI*. This unique approach has not only helped us in understanding the relationship between these genes but may also enable us to obtain an insecticidal lectin carrying insecticidal activity to several insects pests (for which even Bt proteins have been reported to be ineffective), apart from being non-toxic for man, mammals and birds in the future.

Highlights

- Insect pests cause immense destruction and great losses to many of the important crop plants. Bt proteins have although proved as potential candidates against these pests but there are yet no Bt toxins effective against bugs, hoppers or aphids which feed on phloem sap.
- Scientists have reported lectins to possess insecticidal properties against these insect pests but the toxic nature of lectins to man and animals left the researchers in a dilemma.
- Studies are conducted on *LECASAI* lectin gene to determine its insecticidal activity and non-toxic nature for man, mammals and birds by elucidating the presence of mannose binding region within the gene sequence.

Keywords: *Bacillus thuringiensis, LECASAI, Allium sativum* I lectin, Mannose Binding lectins

Introduction

In an approach towards increasing the yield and production of crops, the role of insects pests *viz* aphids, brown and green planthoppers in causing destruction to crops resulting in heavy loss cannot be neglected. These insects pests not only causes severe physiological damage to the crops but also acting as vectors for transmitting viruses like rice tungro virus, ragged (RRSV) and grassy (RGSV) stunt virus (Hibino, 1989; 1996; Mochida *et al.*, 1979; Saxena and Khan, 1989) to important crops like rice (known as staple food in many countries). Much research has been conducted and is still in process in order to control these insect pests. In the present scenario, the commercial transgenic plants expressing insecticidal toxins are basically based on the ectopical expression of toxins derived from *Bacillus thuringiensis* (Bt) bacterium. The Bt proteins have shown potential to protect different crops from several insects of lepidopteran and coleopteran class (Barton *et al.*, 1987; Peferoen *et al.*, 1990; Koziel *et al.*, 1993; Fujimoto *et al.*, 1993; Wunn *et al.*, 1996; Nayak *et al.*, 1997) with chewing type of mouth parts. However, there are as yet no Bt toxins to control the insect pests which feed on phloem sap such as bugs, hoppers or aphids (Malone *et al.*, 2008).

Therefore, the search for alternatives to Bt toxins that can be used to achieve resistance against these pests has received a lot of attention now. One of the most promising groups of candidate proteins bearing insecticidal properties against those insect's pests for which Bt gene also has been found ineffective are plant lectins. Plant Lectins are characterized as "Proteins or Glycoproteins of non-immune origin with one or more binding sites per subunit, which can reversibly bind to the specific sugar segments *via* hydrogen bonds & Van Der Waals interactions" (Lis and Sharon, 1998). The great potential of plant lectins in having insecticidal activity against a large array of insect species belonging to the Coleoptera, Homoptera, Hemiptera, Diptera and Lepidoptera order (Gatehouse *et al.*, 1995; Schuler *et al.*, 1998; Carlini and Grossi-de-Sa, 2002) is well known. But inspite of their noble role in imparting plant resistance against these insect pests, the toxic effects of some lectins and their antinutritional properties in man and animals, has the left the researchers in a dilemma to use plant lectins in further research or not. In this regard, the researchers conducted many experiments, the results of which demonstrated that apart from being insecticidal, many plant lectins posses cytotoxic, fungitoxic, anti-insect and anti-nematode properties which are either *in-vitro* or *in-vivo* toxic for higher animals (Oliveira *et al.*, 1994; Peumans and Van Damme, 1995; Oka *et al.*, 1997; Ripoll *et al.*, 2003) including humans but, in some studies on experimental animals (especially) fed on diets containing plant lectins, the evident symptoms found were loss of appetite, decreased body weight and finally death (Liener *et al.*, 1986; Duranti and Gius, 1997; Lajolo and Genovese, 2002).

In this regard, the presence of a conserved mannose binding region in plant lectins belonging to (Mannose binding lectins) MBL superfamilies is a ray of hope as it is believed that the Mannose binding lectins (MMBLs) although bearing insecticidal activities are considered to non-toxic for mammals and birds (Ripoll *et al*., 2003),

In the present investigation, results from *in-silico* analysis, reported that *LECASAI* gene found in genome of garlic (*Allium sativum* L.), shares homology with some other previously cloned lectin genes bearing insecticidal activity. The results of above findings also reported that the gene was found to carry a conserved mannose binding region (the detailed information is indicated in results section) which allow these lectins to be categorised into a superfamily of mannose-binding monocot lectins (Van Damme *et al.*, 1994) and may add advantage to increase use of lectins in researches.

Thus, keeping all these things in mind, the research was conducted with an aim to isolate, characterize this gene at molecular level, conduct *in-silico* analysis to know this gene more and to establish the phylogenetic relationship between *LECASAI* and other cloned lectin genes possessing the similar characteristics features and to investigate the presence of mannose binding region imparting non-toxic nature to lectins against mammals and birds.

Materials and Methods

Collection, preparation of sample from *Allium sativum*

The bulbs of garlic (*Allium sativum)*, were purchased from a local store during the month of November 2011. The samples were kept aseptically, rinsed with sterile (distilled) water and were allowed to dry to remove the moisture. The samples were then packed into air tight poly-bags and frozed immediately in deep freezer (-20°C) to keep them safely until processing for their use in further DNA isolation steps.

Primer designing for isolation of *lectin* gene from *Allium sativum* L. *via* PCR

The *LECASAI* sequence was retrieved from NCBI, U.S.A database and the gene specific primers were designed by Fast-PCR bioinformatics software. The primers compatible in Tm, Size and GC content were selected and the sequence analysis for PCR was conducted through Bioedit, BTI software. The primer sequence was sent for synthesis at Bioserve. The forward and reverse primers obtained from Fast-PCR, were of 22bp and 20bp respectively.

Isolation of Lectin gene from Genomic DNA of Garlic

The Genomic DNA isolation was conducted through CTAB method of DNA isolation (standardized in lab), the pellet obtained was dissolved in 50µl of Autoclaved distilled water, which after purification and Quantification was kept at -20°C for further use. DNA amplification was carried out in a 50 µl of reaction mixture containing 10X PCR buffer, 2.5 mM $MgCl_2$, 10 mM of each dNTP, 1U *Taq* DNA polymerase, 100-1000ng genomic DNA and 100ng/µl of each of two appropriate primers mixtures in a reaction. The reaction comprises 35 cycles with the following conditions - Denaturation at 94°C for 10 min, Denaturation at 94°C for 30 sec, Annealing at 58°C for 45 sec, Primer extension at 72 °C for 1 min and Final extension at 72°C for 10 min. The primers for the coding regions of *LECASAI* were Forward primer-"LEC1F", Sequence 5'-ACTACTTCATCTCCTAAACTAA-3', Reverse primer -"LEC R", Sequence 5'- ACCAGCAAACGGTGACTTAA-3'.

Competent cell preparation, ligation and Transformation.

PCR amplified product was eluted from agarose gel using gel extraction kit (Qiagen Inc, USA). Eluted DNA fragment was ligated to pTZ57RT cloning vector (size 2.8kb) at 4°C with overnight incubation. The recombinant construct was transformed to *E. coli* DH5α competent cells. Transformants were plated on LB agar supplemented with X-gal/IPTG and ampicillin. Recombinant clones were screened based on blue/white colour colony selection. The presence of correct insert in the recombinant clone was done by isolation of Recombinant Plasmid DNA isolation followed by confirmation by Colony PCR and Restriction enzymes digestion with release of 1029 bp insert by digestion of plasmid with EcoRI and Hind III (Fig 3.6). Restriction analysis of the recombinant clone in this case yielded correct restriction pattern. The recombinant clone was sent for sequencing at Bioserve. The nucleotide sequence obtained was submitted to GenBank, NCBI where it was assigned with the accession number JX561228. The sequence was then blast searched to elucidate the sequence homologues in order to determine the ancestral relationship of the gene with other lectin genes and to investigate its nature in the presence of insecticidal activity. The prediction of ORFs, size and positions, nucleotide translation was performed in order to calculate the theoretical pI and molecular mass of *LECASAI* sequence, and the presence of mannose binding region in the gene of interest.

Results and Discussion

In-Silico analysis of LECASAI *sequence*

The *LECASAI* gene sequence was retrieved from NCBI sequence database, USA and was BLAST searched. The results indicated that the gene shares Maximum percent identity with *Allium sativum LECASAI* and other lectins belonging to various Allium species (Table 1).

Table 1: Blast results indicating the % identity of *LECASAI* with other lectin genes.

	Accession nos	% Identity	Accession nos	% Identity
(1)	M85176.1	95%	(8) EU252577.1	91%
(2)	M85174.1	94%	(9) EU083313.1	91%
(3)	M85177.1	93%	(10) DQ640308.1	91%
(4)	M85175.1	92%	(11) DQ202395.1	91%
(5)	M85172.1	95%	(12) DQ083542.1	91%
(6)	M85171.1	92%	(13) EU083312.1	91%
(7)	M85173.1	91%	AY866499.1	91%

Isolation of *LECASAI* from Garlic

The two sets of primers"Lec1F and LecR", were designed, synthesized and used to amplify the gene of interest. According to the results obtained, the primers were found to be compatible (Table 2) to amplify the 1029bp of band thereby spanning the region of the gene of interest (Fig 1).

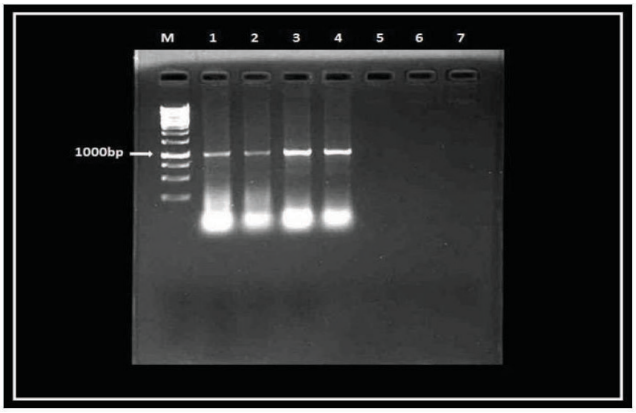

Fig. 1: PCR based amplification of *LECASAI* gene isolated from genomic DNA of *Allium* (from left to right: M-Marker, 1-4 Lec1F, LecR).

Table 2. Results of PCR amplification obtained from specific gene Primer set.

S.No.	Primers	Expected size	Observed Size
(1)	Lec1F, LecR	1029bp	+.

Cloning of PCR amplified product into cloning vector

The PCR amplified and eluted band of 1029bp was then ligated into pTZ57R/T cloning vector of size 2.8kb and were then transformed into *E. coli* DH5α cells. The presence of the cloned fragment was interpreted by blue and white screening technique. The white colonies obtained indicated the positive results while the blue colonies indicated false positives. The plasmid DNA (total size of 3.8kb) was isolated (Fig 2) from the white colonies which gave an idea of the presence of the cloned gene.

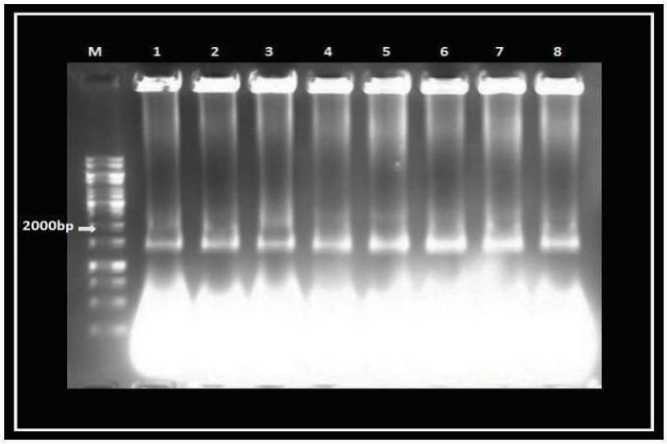

Fig. 2: Electrophoresis results of plasmid DNA containing gene of interest isolated from pTZ57R/T vector (from left to right: M-Marker; 1-8 plasmid DNA with insert).

Confirmation of gene cloned into cloning vector

The colonies were subjected to colony PCR and were used as a template using the same set of primers in a 50ìl colony PCR reaction. The amplicons of 1029bp size, confirmed the presence the gene of interest in the recombinant vector (Fig 3).

Restriction of plasmid with EcoRI (*Escherichia coli*) and Hind III (*Haemophilus Influenzae)* yielded two fragments of 3.8kb (total size of plasmid) and 1029bp (insert size) with the generation of sticky ends at both the sites (Fig 4). The recombinant clones confirmed with the presence of gene of interest was sent for sequencing at Bioserve ompany.

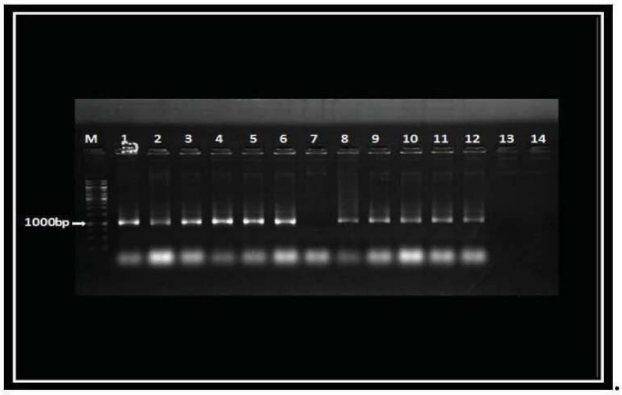

Fig 3. Colony PCR results (from left to right: M-Marker; 7 negative control; 1-6, 8-12 gene of interest in cloned plasmid amplified with Lec1F, LecR primer set)

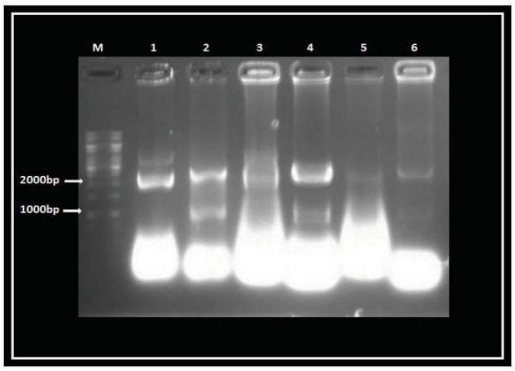

Fig. 4: Results obtained from Restriction digestion (from left to right: M-Marker; 1,3,5 plasmids from positive clones; 2,4,6 plasmid vector and cloned gene of interest).

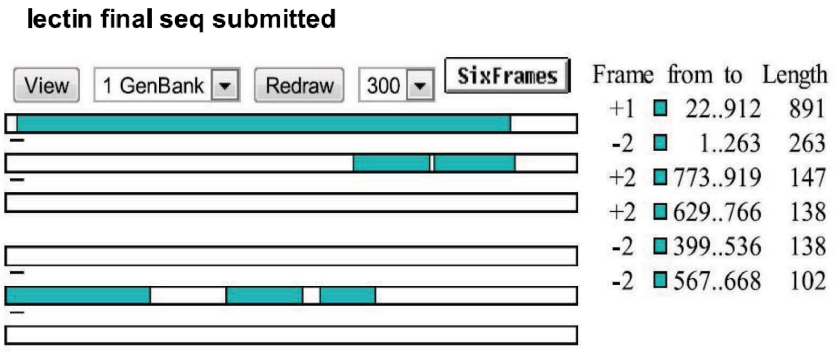

Fig. 5: *In-silico* analysis - the information about the size and position of different ORF's present in the *LECASAI* query sequence.

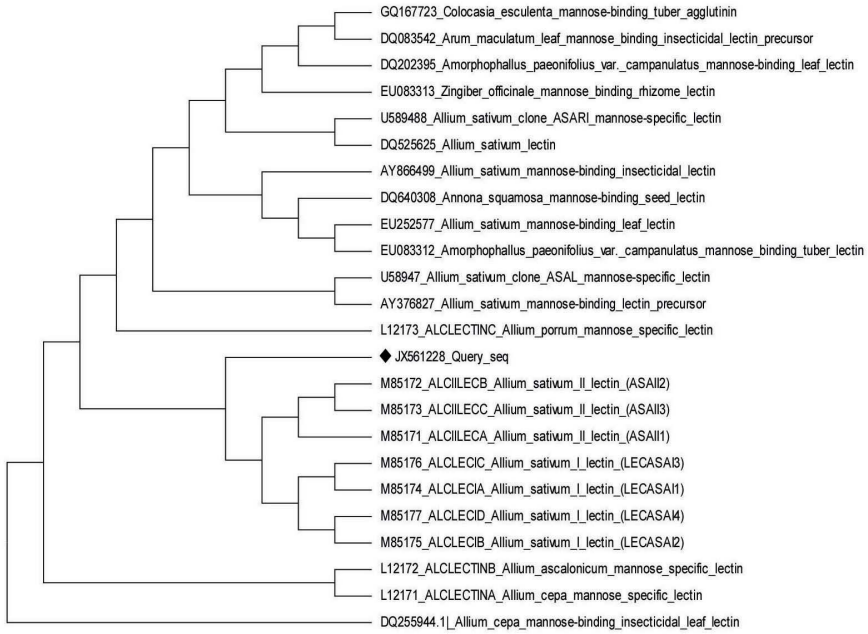

Fig. 6: *In-silico* analysis: Phylogeny of *LECASAI* query sequence (Genbank accession number: JX561228) with other lectins. The phylogenetic tree is based on the nucleotide sequences of previously cloned lectin genes, obtained from BLAST results at NCBI.

In-silico analysis of seaquences

Bioinformatics analysis of 1029bp query sequence was conducted. The results obtained are indicated below. The full length nucleotide sequence of *LECASAI* obtained was Blast, Tblastx searched for the generation of sequence identity matrix and phylogenetic tree construction to see the sequence homology of *LECASAI* with other lectins in order to explore the presence of the insecticidal activity (Fig 6) and non toxic activity, indicating the presence of mannose binding region (Fig 10) within the query sequence. The query sequence was also analyzed in detail with respect to its ORF's (Fig 5), putative start/stop codon, pI value, Mwt, Multiple sequence alignment (Fig 9), Interproscan (Fig 8) and was later subjected to search the conserved domains (Fig 7) to know the gene more and to detect and get an idea/positions of the presence of the conserved mannose binding region in the query sequence.

The molecular weight of the full length *LECASAI Allium sativum* lectin gene sequence was found to be 84395.06 Da. The pI (Isoelectric point value) of *LECASAI* gene query sequence obtained was 5.07. The pI and Molecular weight was calculated using the Expasy Bioinformatics resource portal. The sequence was later used to obtain ORFs. Six ORFs were found on the *LECASAI* query sequence, out of them the ORF (frame +1) was found to be the largest ORF among all other ORF which starts with a start codon ATG from 22 position and ends with a stop codon TAG at 912 on plus strand, the total length of ORF comprise is of 891 bp.

The Multiple sequence alignment was done to detect and compare the conserved mannose binding region of the query sequence with that in other MBLs. The Results clearly characterized the query sequence to be a Bulb-type mannose-specific lectin, the domain of which consists of 3-fold internal repeat (beta-prism architecture). The

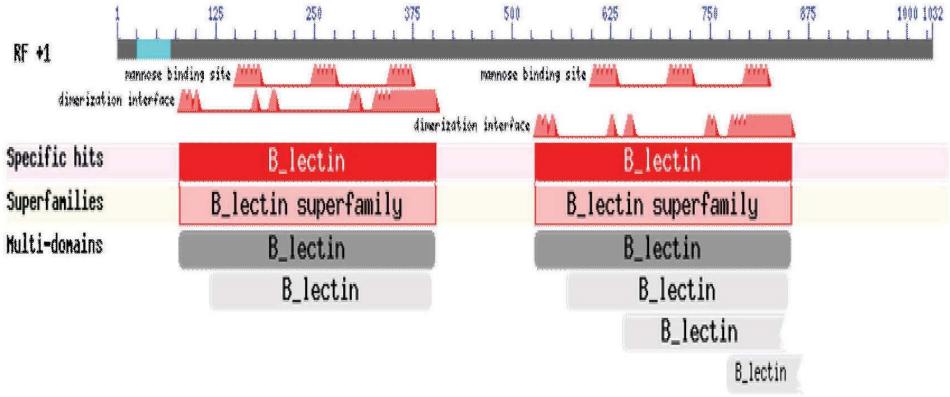

Fig. 7: *In-silico* analysis- Conserved mannose binding domains, multi-domains, region and their occurrence within the *LECASAI* sequence.

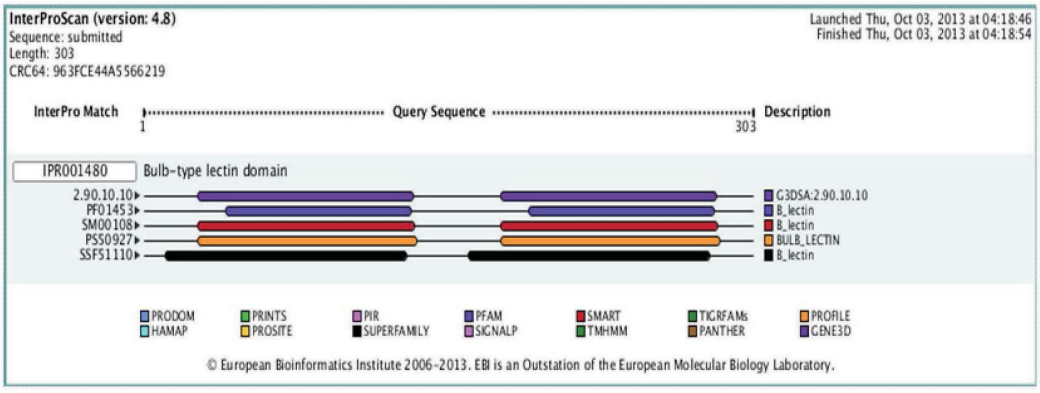

Fig. 8: *In-silico* analysis - Interproscan results showing the matched amino-acid sequences of mannose binding regions in *LECASAI* sequence with other *Allium* species seaqences (submitted in a total of 14 databases).

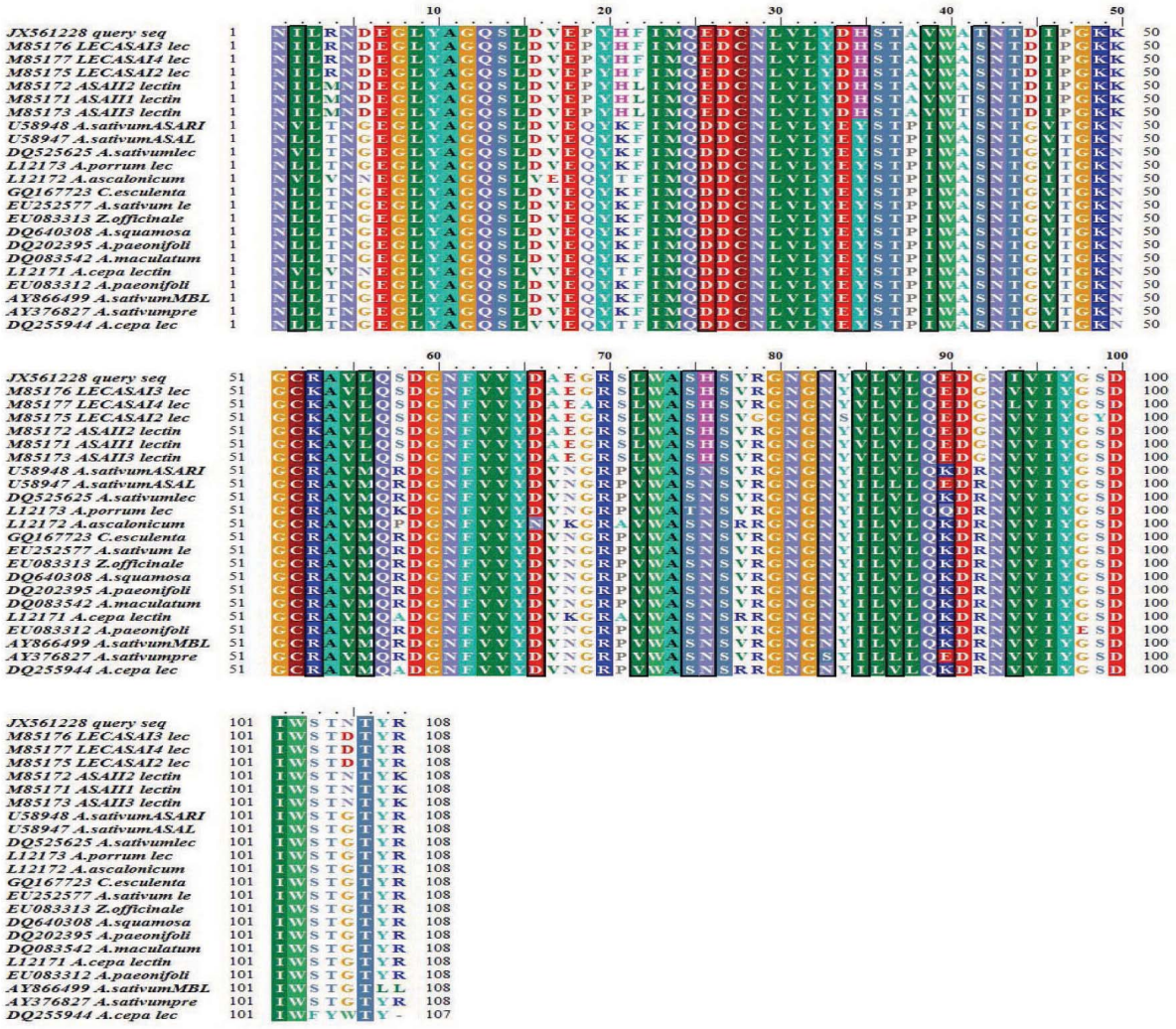

Fig. 9: *In-silico* analysis - Multiple sequence alignment (MCS) indicating the presence of consensus sequence motif **QXDXNXVXY** responsible for detection of alpha-D-mannose binding region.

presence of consensus sequence motif QXDXNXVXY was found to be involved in the recognition of alpha-D-mannose binding region. The full length *LECASAI* gene sequence was then used to locate the positions of conserved mannose binding domains *via* Conserved Domain Search Service (CD Search) tool at NCBI. According to the results obtained the mannose binding region was found to be present in a range of 150-375bp and 600-825 bp within the query sequence. The triangles represent the amino acids comprising conserved regions/sites mapped from conserved domain annotations. The specific hits/ multi domains/B-lectin superfamilies region comprise a region between 75-400bp and 525-850 bp while the dimerization interface was occupied from 75-400bp and 525-850bp. However, the low complexity region was found between 25-75 base pairs. Thus, the query sequence was confirmed to belong to the super family of Mannose binding Lectins.

The phylogenetic tree of the *Alliaceae* lectins reveals some interesting evolutionary relationships. According to the results obtained in dendrogram tree analysis lectins were divided into three major groups. One group contained lectins viz. *ASAI*, *LECASAI* all derived from garlic (*A. sativum*). It highlighted and confirmed that the query sequence was grouped together with *LECASAI* and *ASA* genes found in garlic (from which the query sequence was actually obtained). The second group comprises of lectins

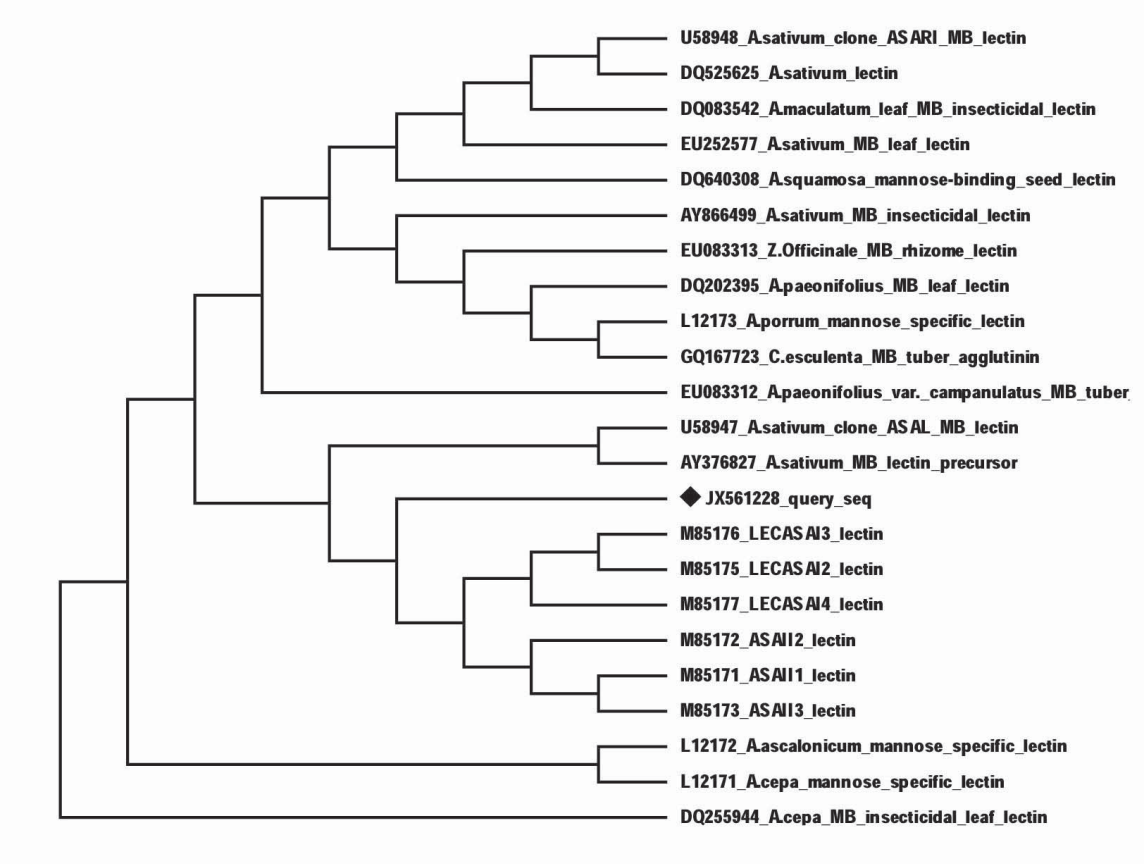

Figure 10. *In-silico* analysis. Phylogeny of amino acid sequences encoding mannose binding regions in *LECASAI* and other previously cloned lectin genes from *Allium* species. The tree was constructed using simultaneous alignment and phylogeny program (Mega4).

from leaf, rhizome, seed and tuber (with mannose binding feature in common). The third group purely contained mannose specific lectins derived from *A. cepa* species. Out of these lectins most of the lectins broadly have already been reported to belong to the superfamily of mannose binding lectins and some with being insecticidal too. Thus, it can be indicated that all these groups of lectins were derived from a common ancestor during evolution, suggesting that these lectins shared a common evolutionary ancestral relationship. A similar result were observed in evolutionary relationship of lectin gene with deduced amino acid sequences for the presence of mannose binding regions which showed almost similar results as mentioned above and confirmed that the *A. sativum* query sequence is highly related to *LECASAI* & *ASAII* (all derived from *A. sativum*), followed by lectins present in different plant parts of *A. sativum* or other Allium species including *Allium cepa*.

This study, *LECASAI* bulb specific gene which refers to *Allium sativum* agglutinin I (ASAI) - a heterodimeric bulb lectin composed of 11.5 kDa subunit (Smeets *et al.*, 1997) which through bioinformatics analysis was found to be homologous to lectins bearing insecticidal activity and also to those lectin genes carrying the conserved mannose binding region. The bulb was found to be a bulb Mannose binding Lectin (MBL) and the plant lectin gene *LECASAI* was predicted to carry insecticidal activity by being non toxic for mammals.

The results from BLAST showed a high degree of similarity of *LECASAI* with other lectins present in different Allium species carrying insecticidal activity. The results showed that *LECASAI* gene shares a percent homology of 95% 94%, 93%, 92%, 91% with published *LECASAI3,ASAII2* (Genbank Accession no: M85176,M85172), *LECASAI1* (M85174), *LECASAI4* (M85177), *LECASAI2,ASAII1*

(M85175,M85171) and ASAII3 (M85173). A relatively less percent homology of 88% was identified with *Allium cepa* lectin (DQ255944).

The region (translated protein) in query sequence specific for coding bulb-type mannose-specific lectin containing a consensus sequence motif QXDXNXVXY involved in alpha-D-mannose recognition was scanned for InterProScan results (the software allows us to scan the sequence for matches against the InterPro collection of 14 protein signature databases), in order to confirm the presence of lectin domain/mannose binding region in the query sequence. The results found were satisfactory, clearly indicating and confirming the presence of bulb-type lectin domain region within the query sequence. Interestingly, the results from tblastx and the phylogenetic tree analysis of *LECASAI* gene (for confirmation of the presence of conserved mannose binding region) were also found exactly same as that of BLAST results. The query sequence also showed a higher degree of homology with respect to mannose binding regions with other MBLs too.

Conclusion

Thus, taking into consideration the enigmatic nature of lectins in sharing similar overall characteristics features despite varying identities and the potential of plant lectins in providing plants resistance against various insect pests, the search for more lectin genes has became a necessity. It has been found that the mannose binding garlic lectins are closely related proteins sharing conserved mannose binding regions (a feature of most mannose binding superfamilies of lectin), found to be encoded by homologues gene sequences. Thus, it can be concluded that the present research enabled in elucidating the presence of conserved mannose binding regions and the relationship of *LECASAI* gene with the other previously cloned lectin genes already believed to be carrying insecticidal activity against various insect pests. It can be stated that the *LECASAI* gene carries insecticidal activity apart from being nontoxic to mammals and that the gene may help in understanding the roles, distribution of lectins in the environment. Above all, In future this gene can be used in controlling pests (which feeds the phloem sap) and some fungal diseases without harming the human health.

Accession number

The sequence of *LECASAI* gene was submitted to GenBank *via* Bankit where it was assigned the accession number JX561228 by National Center for Biotechnology Information (NCBI), U.S.A.

Acknowledgements

The Author is thankful to the Council of Science and Technology, (U.P-India), Department of Biochemistry and Physiology, Immunology and Defense Mechanism, Sardar Vallabhbhai Patel university of Agriculture and Technology, Meerut for providing facilities to undergo the study. The help of Miss. Rosy Rani, Miss. Juhi Bhardwaj, Mr. Amit Kumar (SRFs) and Mr. Harish Chandra is sincerely acknowledged.

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