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Molecular Characterization of Snowdrop Lectin (*GNA*) and its Comparison with Reported Lectin Sequences of Amaryllidaceae

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Abstract

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Plant lectins have become efficient sources of insect resistance in crops. The present study was conducted to identify, amplify, clone and characterize the plant lectin gene *GNA*. The lectin, present in *Galanthus nivalis* (snowdrop), is an agglutinin toxic to hemiptera. The attempt was made to elucidate the relationship of the lectin gene *trGNA* (*GNA* isolated and characterized from Turkey) with other previously cloned lectins having insecticidal activity and to ensure the presence of the conserved mannose-binding region/site in the gene sequence. The full-length cDNA of *trGNA* was 477 bp that contained a 333 bp open reading frame encoding 157 amino acid proteins with 23 amino acids of signal peptide. BLAST results showed that *trGNA* has 89–97% similarity with previously reported *GNA* sequences while it has 84–96% similarity with earlier reported *GNA* protein sequences. No intron was detected within the region of genomic sequence corresponding to *trGNA* full-length cDNA. According to the search results from the NCBI (National Centrer for Biotechnology Information database), *trGNA* from *Galanthus nivalis* is most similar to the previously reported lectin sequences of *Narcissus tazetta* with a similarity percentage of 87%. The obtained results are useful for engineering of plants with enhanced insecticidal activity against chewing and sucking insects, causing crop pests. In addition, medical application of lectins may also be considered.

Keywords: bioinformatics; biotic stress; *Galanthus nivalis*; gene cloning; gene sequence; insecticidal activity

According to estimations 13% of the incurred crop losses belong to the damage caused by insect pests (Gatehouse *et al*. 1992; Bakhsh *et al*. 2015). Although the efficacy of Bt (*Bacillus thuringiensis*) toxins against many pests, especially lepidopterans, has been established, no considerable effect of Bt toxins on phloem-feeding pest bugs, hoppers or aphids have been reported yet that have emerged as primary pests.

Plant lectins are being considered as a promising strategy to encode resistance against sap-sucking homopteran insects. Lectins inhibit the nutrient absorption or disrupt the midgut cells by stimulating endocytosis and possibly other toxic metabolites present in the midgut (Czapla & Lang 1990; reviewed in Bakhsh *et al.* 2015). Lectins have variant binding specificities causing different toxic impacts. Different monocot mannose-binding lectins have been isolated from the families Amaryllidaceae, Araceae, Asparagaceae, Smilacaceae, Taxaceae, Iridaceae, and Orchidaceae.

Mannose-binding *Galanthus nivalis* agglutinin is a tetrameric lectin protein in which each subunit is a 12.5 kDa polypeptide being synthesized on the rough

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endoplasmic reticulum. There are three conserved amino acid sequence motifs (QXDXVXNXY) existing in each subunit primarily reported by Van Damme *et al*. (1991, 1992, 1993a, b, 1995). As the first lectin isolated from Amaryllidaceae, *GNA* lectin is different from other monocot and dicot lectins with respect to its molecular structure and very specific carbohydrate binding property (Van Damme *et al*. 1987). *GNA* lectin such as garlic, onion and leek lectins is toxic to Hemiptera insects while no harmful impact on the mammalian oral system has been proved (Peumans & Van Damme 1996). *GNA* exhibits an exclusive specificity towards mannose when it binds to the terminal α-1-3-linked mannose; due to the absence of α-1-3-linked mannose in the mammalian brush border of the small intestine membrane and based on rat model studies it is not toxic to humans (Pusztai *et al*. 1995, 1996; Vandenborre *et al*. 2011).

The present study was conducted with an aim to isolate and characterize the plant lectin gene from snowdrop plants collected from areas of their natural distribution in Turkey. Furthermore, molecular and in-silico analysis was conducted to learn more about this gene and to establish the phylogenetic relationship between the isolated gene and earlier reported Amaryllidaceae lectin genes.

MATERIAL AND METHODS

Plant material. Snowdrop (*Galanthus nivalis* L.) plants were collected during the winter season from the domestic distribution areas of snowdrop in western hills surrounding the city of Ankara, in the Central Anatolia Region, Turkey; fresh leaves were stored at –80°C after immediate freezing by liquid nitrogen treatment.

Chemicals and reagents. TRIzol® RNA isolation reagents, RNase *AWAY*™ decontamination reagent enzymes, cDNA synthesis, plasmid mini preparation, calf intestinal alkaline phosphatase (CIAP) and gel extraction kits were supplied by Thermo Fisher Scientific Inc., Waltham, USA; pGEM®-T Easy Vector was supplied by Promega Corp., Madison, USA; diethyl pyrocarbonate was supplied by Sigma-Aldrich Corp., St. Louis, USA.

Primer designing. The previously reported *GNA* sequences were retrieved from NCBI database. All the reported sequences (M55555.1, M55556.1, M55557.1, M55558.1, M55559.1, AF413083.1, AF413084.1, AF413085.1, AF413086.1 and AF413087.1) were aligned to find the most similar regions upstream and downstream the selected gene sequence. Selected 19 and 20 bp, respectively, forward and reverse primers were analysed by OLIGO 7 Primer Analysis Software (Molecular Biology Insights Inc., Colorado Springs, USA) to check hairpin structure, Tm, GC%, primer dimer and other requisite properties of primers. Designed primers were ordered to synthesis (Integrated DNA Technologies Inc., Coralville, USA).

Isolation of *GNA* lectin gene. Frozen plant leaf samples of snowdrop were subjected to total cellular RNA isolation following the TRIzol® RNA isolation reagent product manual. Using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) quality and quantity of the isolated RNA were evaluated. Approximately 1 µg of the fresh isolated total cellular RNA was converted to cDNA following the protocol as described in the manual of RevertAid First Strand cDNA Synthesis Kit. cDNA was used as template to amplify *GNA* in PCR (polymerase chain reaction). PCR was conducted using gene specific forward and reverse primers, 5'ATGGCTAAGGCAAGTCTCC3' and 5'TCATTACTTTGCCGTCACAA3' containing flanking sites of *Xho*I restriction enzyme (the incorporation of *Xho*I site was planned to be further used for cloning fragments into a binary vector). PCR was carried out by using Dream Taq DNA Polymerase, preceded by 3 min of initial denaturation (95°C). Subsequently 30 s of (95°C) denaturation, 30 s of (58°C) annealing and 1 min of (72°C) extension steps were programed for each of the 35 cycles applied, ending with 10 min of final extension at 72°C.

Molecular cloning and characterization of *GNA* lectin gene. To purify the *GNA* gene, PCR product was loaded in 1% (w/v) agarose gel. Precisely, the separated band of interest was excised from the gel and purified by Thermo Scientific Gene JET Gel Extraction Kit. The purified fragment was quantified and ligated into pGEM®-T Easy Vector (size 3.015 kb) and further transferred to JM109 *E.coli* competent cells. To screen the positive colonies, ampicillin selection and blue white test were applied. Some of the white bacterial colonies were selected and were subjected to colony PCR. The positive colonies were grown in LB broth (Duchefa Biochemie B.V., Haarlem, Netherlands) medium containing an appropriate antibiotic for overnight and using the plasmid mini preparation kit, plasmids were extracted. The restriction digestion reaction was conducted using the *Xho*I enzyme to excise a fragment of the *GNA* gene from a recombinant plasmid. The recombinant

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plasmid was sent for sequencing (RefGen Corp. ,Ltd., Ankara, Turkey).

The obtained nucleotide sequence (called *trGNA* gene) was then blasted to elucidate the sequence homologues in order to determine the ancestral relationship of the gene with other lectin genes. The prediction of ORFs (open reading frame), size and positions, nucleotide translation and presence of mannose-binding region was performed. The *trGNA* gene sequence has been planned to be submitted to GenBank, NCBI to assign it an accession number.

RESULTS AND DISCUSSION

The forward and reverse primers contained flanking sites of *Xho*I. The PCR amplification results showed a 477 bp fragment of *GNA* genes using cDNA template from snowdrop (Figure 1A). The PCR fragment of 477 bp was eluted from agarose gel, purified and used as an insert to be ligated into pGEM®-T Easy Vector (3.015 kb). Transformation of the ligated product (*GNA* gene + pGEM®-T Easy Vector) was performed in JM109 *E.coli* cells. The presence of the cloned fragment was interpreted by a blue and white screening technique. The random white colony selection from LB plates showed that selected white colonies contained the gene of interest as revealed by colony PCR (Figure 1B). Restriction of the recombinant plasmid with *Xho*I yielded two fragments of 3.015 kb (plasmid) and 477 bp (insert size) with the generation of sticky ends at both sites (Figure 1C).

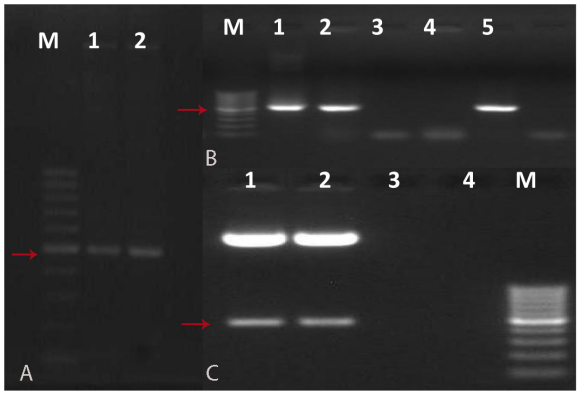


Figure 1. Amplification, cloning and further confirmation of *trGNA* (477 bp) in pGEM®-T Easy Vector: (A) amplification of *trGNA* from cDNA template of snowdrop leaf, (B) colony PCR to confirm positive clones of *trGNA*, (C) restriction digestion of the vector to excise *trGNA* gene; GeneRuler100 bp DNA ladder was used to confirm the desired fragments

Table 1. BLAST results indicating the identity percentage of *trGNA* with other *GNA* sequences reported earlier

NCBI accession No.	Identity (%)	Query cover in blasting (%)
M55556.1	97	100
AF413086.1	91	100
AF413083.1	90	100
AF413087.1	90	100
AF413085.1	90	100
AF413084.1	89	100
M55558.1	90	98
M55557.1	88	97
M55559.1	91	78
JQ898015.1	96	66
M55555.1	89	80

The recombinant clones with the confirmed presence of the gene of interest were sent for sequencing.

The obtained sequence was called *trGNA*. Bioinformatics analysis of 477 bp query sequence was conducted. The full-length nucleotide sequence of *GNA* gene obtained was blasted, and subjected to the generation of sequence identity matrix and phylogenetic tree construction to see the sequence homology of *trGNA* with previously reported *GNA* sequences

***trGNA* DNA sequence**

ATGGCTAAGGCAAGTCTCCTCATTTTGGCCACCATCTTCCTTGGTGTCATCACACC
ATCTTGTCTGAGTGACAATATTTTGTACTCCGGTGAGACTCTCTCTGCAGGGGAAT
TTCTCAACTACGGAAGTTTCATTTTTATCATGCAAGAGGACTGTAATCTGGTCTTG
TACGACGTCGACAAGCCGATCTGGGCAACAAACACGGGTGGCCTCTCCCGTAGCT
GCTCCCTCAGCATGCAGACTGATGGGAACCTCGTGGTGTACAACCCATCGAACAA
ACTGATTTGGGCAAGCAACACTGGAGGCCAAAATGGGAATTACGTGTGCACCCT
ACAGAAGGATCGGAACGTTGTGATCTACGGAACTGATCGTTGGGCTACTGGAACT
CACACCGGACTTGTTGGAATTCCCGCATCGCCACCCTCAGAGAAATATCCTACTG
CTGGAAAGATAAAGCTTGTGACGGCAAAGTAATGA

***trGNA* Protein Sequence**

MAKASLLILATIFLGVITPSCLSDNILYSGETLSAGEFLNYGSFIFIMQEDCNLVLYDVD
KPIWATNTGGLSRSCSLSMQTDGNLVVYNPSNKLIWASNTGGQNGNYVCTLQKDRN
VVIYGTDRWATGTHTGLVGIPASPPSEKYPTAGKIKLVTAK**

Figure 2. The nucleotide and predicted amino acid sequences of *GNA* lectin (*trGNA*); the full length of 477 bp with a 333 bp open reading frame encoding a putative peptide of 157 amino acids; the residues comprising a 23-amino acid signal peptide are underlined; the conserved regions that make up the mannose-binding sites are highlighted in grey; asterisk indicates the position of the stop codon

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(Table 1). BLAST results showed that *trGNA* has 89–97% similarity with previously reported *GNA* sequences while it has 84–96% similarity with earlier reported *GNA* protein sequences. The full-length cDNA of *trGNA* was 477 bp that contained 333 bp open reading frame encoding 157 amino acid proteins with 23 amino acids of signal peptide (Figure 2). The DNA sequence of *trGNA* is shown in Figure 2.

The query sequence was also analysed in detail with respect to its ORF's, putative start/stop codon, and multiple alignment of the DNA and protein sequence (Figures 2–5). The sequence was subjected to search the conserved domains to learn more about the gene and to detect and get idea/positions of the presence of the conserved mannose-binding region in the query sequence.

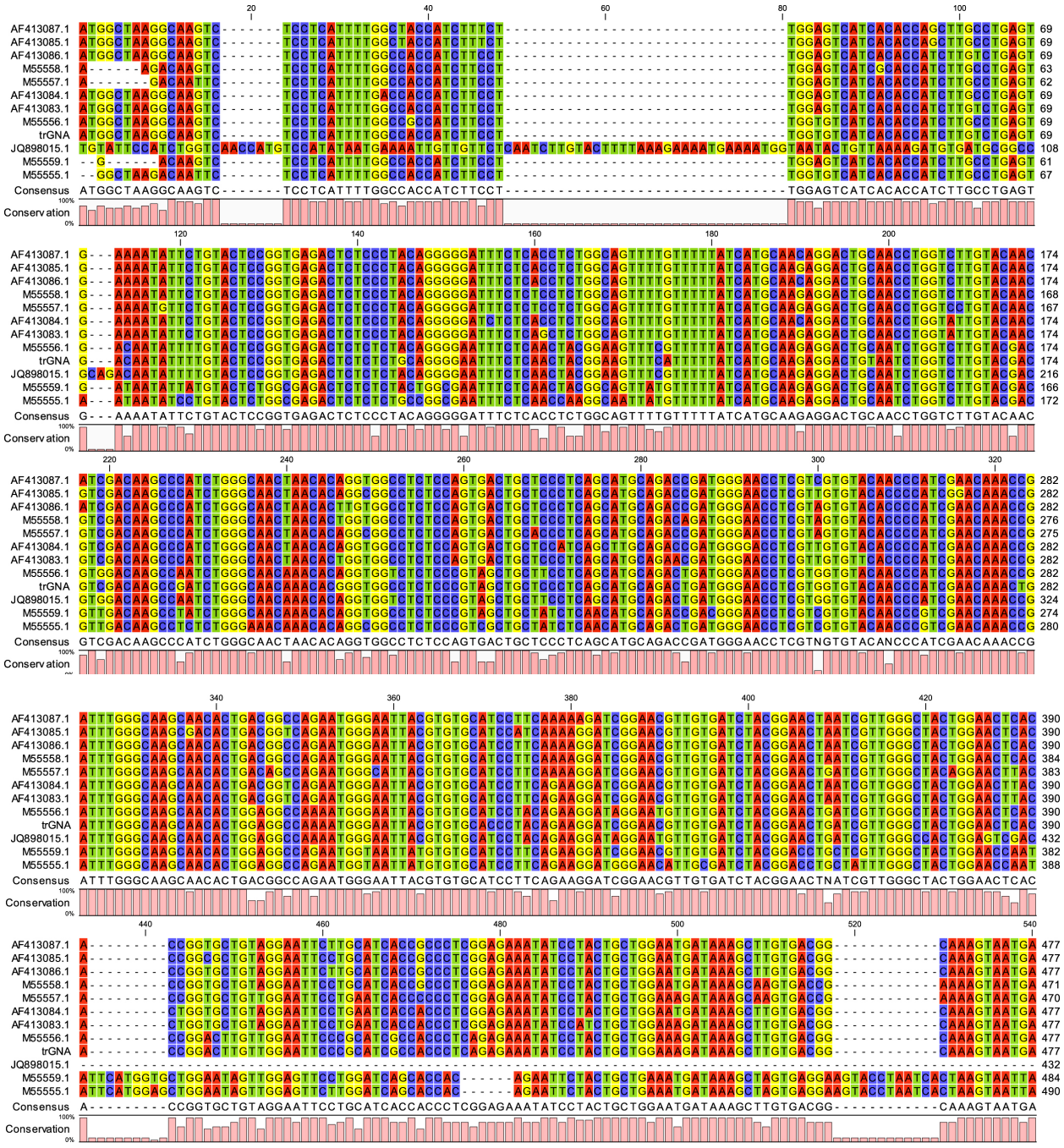


Figure 3. In-silico analysis – multiple DNA sequence alignment indicating the presence of conserved sequence regions (performed with the CLC Sequence Viewer 7.6.1 software)

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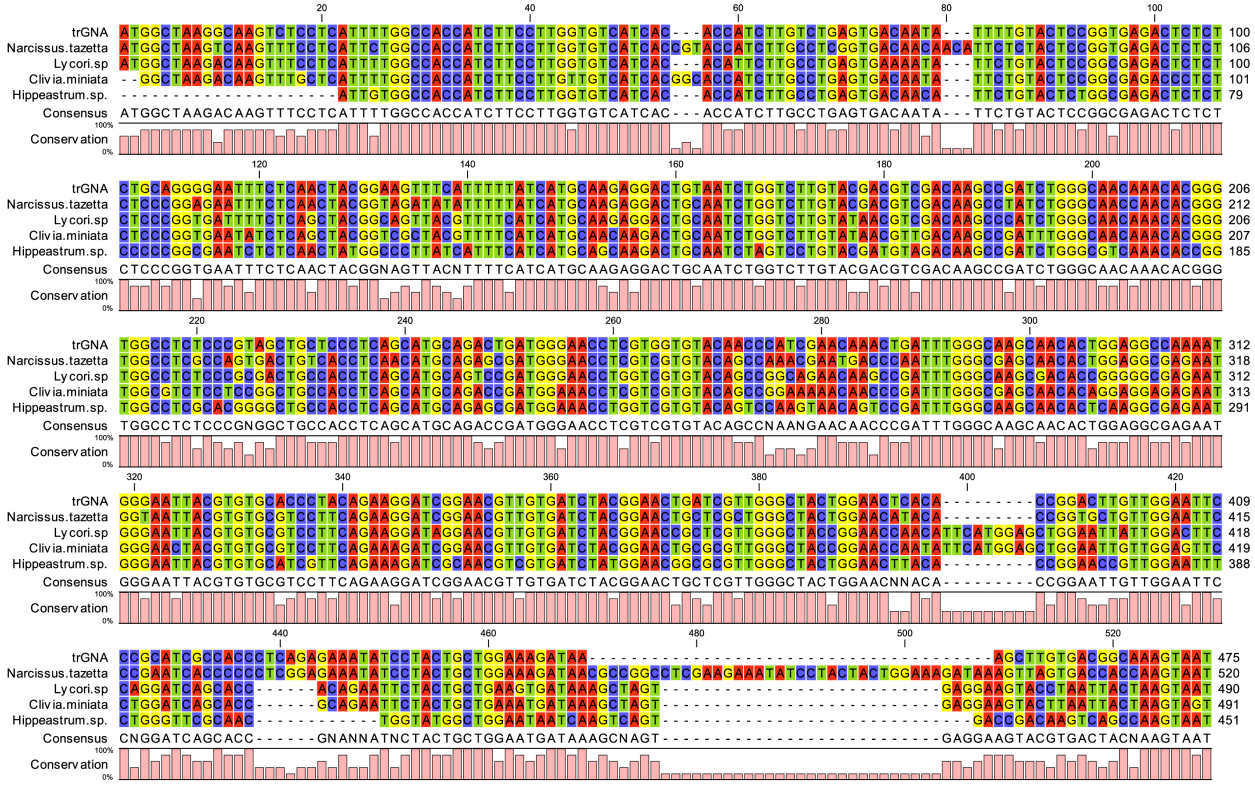


Figure 4. In-silico analysis – multiple DNA sequence alignment indicating the presence of conserved sequence regions in some different Amaryllidaceae mannose-binding agglutinin lectins including *Galanthus nivalis, Narcissus tazetta, Lycoris radiata*, *Clivia miniata*, *Allium cepa*, *Allium sativum* (performed with the CLC Sequence Viewer 7.6.1 software)

Multiple alignment of the protein sequence of *trGNA* with those of mannose-binding lectins from Amaryllidaceae was performed with the CLC Sequence Viewer 7.6.1 (CLC Bio-Qiagen, Aarhus, Denmark) using the published mannose-binding lectin sequences (Figure 5). Gaps were introduced for optimal alignment and maximal similarities between all compared sequences.

The phylogenetic tree is based on the nucleotide sequences of previously cloned *GNA* genes, obtained from BLAST results at NCBI (Figure 6). The results from NCBI BLAST showed a high degree of similarity

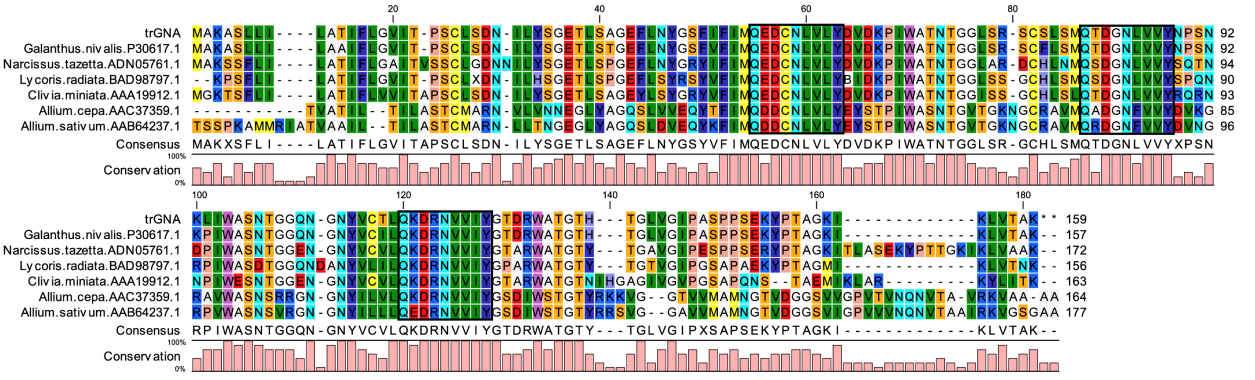


Figure 5. In-silico analysis – multiple protein sequence alignment indicating the presence of consensus sequence motif responsible for detection of α-D-mannose binding region (boxed in black) in different Amaryllidaceae species (performed with the CLC Sequence Viewer 7.6.1 software)

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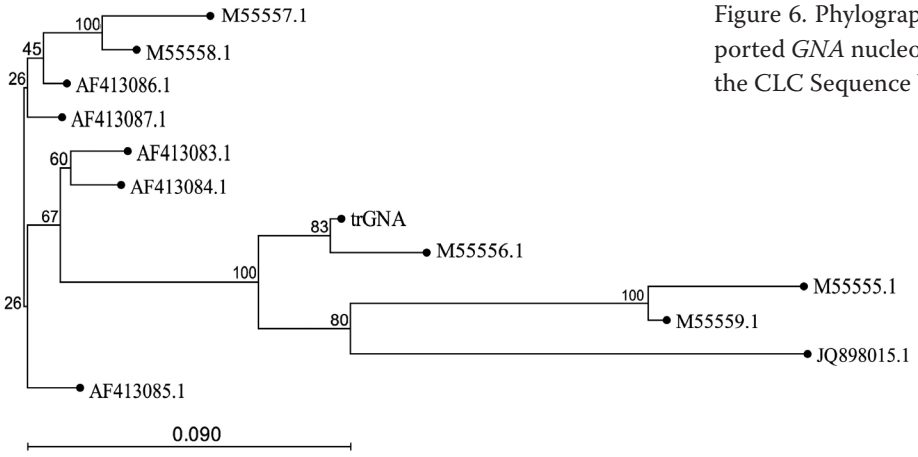


Figure 6. Phylograph of *trGNA* with previously reported *GNA* nucleotide sequences (performed with the CLC Sequence Viewer 7.6.1 software)

of *trGNA* with other reported *GNA* lectins. According to the protein sequence BLAST results of the *trGNA* and some Amaryllidaceae mannose-binding lectins there is a close relationship which has been presented as a phylogenetic tree in Figure 7.

GNA lectin was isolated and characterized by Van Damme *et al*. (1987) and it has been used widely by researchers as an insecticidal gene since then. Primary structure of the gene was introduced in 1991 (Van Damme *et al*.). According to the homology blasting results of the present study there is a nucleotide identity of 88-90% and amino acid identity of 76–96% with sequences reported by Van Damme *et al*. (1991). The isolated *trGNA* protein sequence was compared with some published sequences of Amaryllidaceae species. Homology blasting with the previously published lectin sequences of *Narcissus tazetta* (Gao *et al*. 2011), *Lycoris radiata* (Hayashi *et al*. 2005), *Clivia miniata* (Van Damme *et al*. 1994), *Allium cepa* (Van Damme *et al*. 1993a) and *Allium sativum* (Smeets *et al*. 1997) resulted in 82, 79, 75, 51 and 48% amino acid sequence identity, respectively.

According to the alignment table study, highly conserved motifs of QXDXNXVXY were confirmed in three positions on amino acid sequences of the studied Amaryllidaceae lectins, remark the mannose-binding protein type in all the aforementioned lectin sequences. The results showed that the *trGNA* gene shares a percent homology of 97, 91, 90, 90, 90, 89, 90, 88, 91, 96 and 89% with already reported *GNA* sequences, in NCBI, M55556.1, AF413086.1, AF413083.1, AF413087.1, AF413085.1, AF413084.1, M55558.1, M55557.1, M55559.1, JQ898015.1 and M55555.1, respectively. Comparing the similarity between the isolated *trGNA* and other lectin gene sequences revealed the similarity of 85–87% and 83% of *trGNA* with *Narcissus tazetta* and *Lycoris radiata* agglutinin lectin genes, respectively. According to the results of NCBI nucleotide blasting, *trGNA* isolated from *Galanthus nivalis* is most similar to the previously reported agglutinin lectin sequences of *Narcissus tazetta* with the similarity percentage of 87 and query cover of 95%.

Thus, taking into consideration the enigmatic nature of lectins in sharing similar overall characteristic 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 become 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

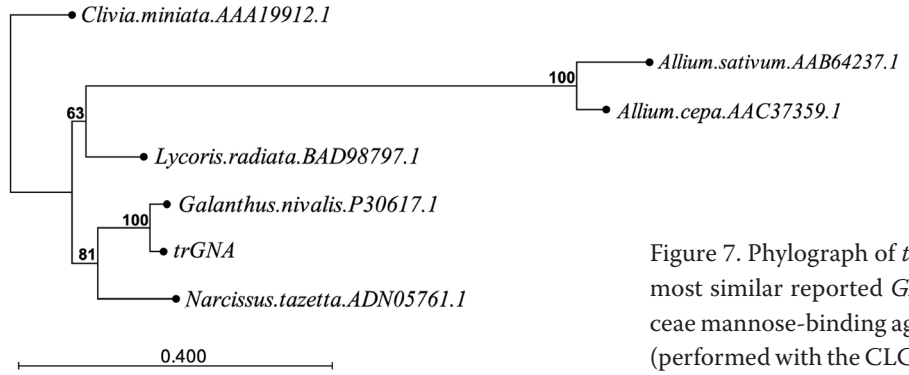


Figure 7. Phylograph of *trGNA* protein sequence with the most similar reported *GNA* and some other Amaryllidaceae mannose-binding agglutinin lectin protein sequences (performed with the CLC Sequence Viewer 7.6.1 software)

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mannose-binding superfamilies of lectin), found to be encoded by homologues gene sequences. Thus, it can be concluded that the present research enabled to elucidate the presence of conserved mannose-binding regions and the relationship of the *trGNA* gene with the other previously cloned lectin genes already believed to be carrying insecticidal activity against various insect pests. Currently we are using *trGNA* in pCAMBIA1301 for controlling sucking pests in cotton.

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