Molecular cloning and characterization of six defensin genes from lentil plant (*Lens culinaris* L.)

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Abstract

Six full-length gene and cDNA sequences of defensin were identified from *Lens culinaris* L. plant. The identified genes and cDNAs were different in length and their coding sequences contained Knot1 functional domain. Phylogenetic analysis classified the identified defensins into two subfamilies. All defensin genes contained only one intron and had extracellular signal peptides. Secondary structures of identified defensins were completely composed of alpha helix and beta strand. Presence of conserved Cys amino acids and disulfide bridges, interaction with defense and signaling proteins and antimicrobial activity were other common features of these peptides. The identified defensins displayed differential expression pattern in the various tissues. The highest expression level of defensins was observed in seed, pod, and root tissues. Defensin 4 was significantly expressed in all examined tissues, whereas the other defensins were only expressed in some tissues. Also, in the fungal and wounding treatments, lentil defensins showed different expression pattern. Defensin 1 was up-regulated in both fungal and wounding treatments. Defensin 4 showed decreased expression level in both fungal and wounding treatments. Defensins 2 and 6 were up-regulated in wounding and fungal treatments, respectively. In this study, for the first time, six defensin genes were isolated and characterized from lentil. Our results highlighted the role of defensins in lentil plant that can be used for future studies.

Keywords Antimicrobial activity · Defensins · Gene isolation · Intron · *Lens culinaris* L

Abbreviations

ANN	Artificial neural network
AMPs	Antimicrobial peptides
CAMP	Collection of anti-microbial peptides
cDNA	Complementary deoxyribonucleic acid

DA	Discriminant analysis
EST	Expressed sequence tag
GRAVY	Grand average of hydropathicity
MW	Molecular weight
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
pI	Isoelectric pH
RF	Random forest
SRA	Sequence Read Archive
SVM	Support vector machine

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Introduction

During their growth and development, plants are simultaneously exposed to various adverse conditions including biotic and abiotic stresses (Jeandroz and Lamotte 2017). Biotic stresses occur as a result of damage done to plants by living organisms, such as bacteria, viruses, fungi, parasites and insects. Plants use several defense mechanisms to deal with these adverse conditions (Roy 2012). The production and secretion of antimicrobial peptides (AMPs) is one

of these mechanisms (Aerts et al. 2008; Castro and Fontes 2005; Goyal and Mattoo 2016). Plant AMPs are a large and diverse group of defense proteins. They are important part of the immune system of plants and animals (Goyal and Mattoo 2016). AMPs are small basic peptides with inhibitory activity against a broad range of pathogens and pests (Vriens et al. 2014). The expression and production of plant AMPs may be continuous (e.g., in storage and reproductive organs) or induced (systemically or locally) (Bowles 1990; Heil and Bostock 2002; Vriens et al. 2014). Based on protein sequence similarity, tertiary structure, conserved cysteine amino acids and disulfide bridge patterns, plant AMPs are divided into eight classes, including lipid transfer proteins, heveins, knottins, cyclotides, thionins, α -hairpinin, snakins and defensins (Tavormina et al. 2015; Tam et al. 2015; Goyal and Mattoo 2016).

Plant defensins are a family of cysteine-rich cationic AMPs. They are small molecules (45–54 amino acid residues) with molecular mass between 5 and 7 kDa. They have eight conserved cysteines that are involved in disulfide bond formation. Defensin AMPs exist in all plant families and play vital biological roles in plant defense responses and growth and development (Cools et al. 2017). Plant defensins are well-studied for their broad-spectrum antimicrobial activity and also for the innate immune system mediatory roles against insects and invading pathogens (Bloch and Richardson 1991; Cools et al. 2017). During recent decades, considerable studies have been performed to identify antimicrobial mode of action of defensins. Different studies have demonstrated that defensin AMPs exert their antimicrobial activity specifically by interaction with membrane compounds, such as lipid receptors, phospholipids and sphingolipids (Vriens et al. 2014; Wilmes et al. 2011; Poon et al. 2014).

So far, many defensins have been isolated and characterized from different plant species.

In the study conducted by Osborn et al., five antibacterial and antifungal defensins were isolated from plants of Asteraceae, Fabaceae, Hippocastanaceae, and Saxifragaceae families (Osborn et al. 1995). The defensin isolated from seeds of *Medicago sativa* plant showed high antifungal activity against *Verticillium dahliae*. Recombinant expression of this peptide in transgenic potato plants provided robust resistance against fungal pathogens (Gao et al. 2000). In other study, four defensin genes were isolated from a native South African Brassicaceae species, *Heliophila coronopifolia*. Isolated defensins showed different antifungal activity against *Fusarium solani* and *Botrytis cinerea* pathogens (De Beer and Vivier 2011). In a similar study, two defensin peptides were isolated from *Vicia faba* that showed inhibitory activity against Gram-negative and Gram-positive bacteria but not the yeasts (Zhang and Lewis 1997). Defensin isolated from *Triticum aestivum* showed antibacterial activity against

phytopathogenic bacteria *Pseudomonas cichorii* (Koike et al. 2002). Finkina et al., isolated a defensin peptide from lentil seeds with an antifungal activity against *Aspergillus niger* (Finkina et al. 2008).

Lentil (*Lens culinaris* L.) is an important legume and delivers affordable source of dietary components and plays a crucial role in alleviating malnutrition (Kumar et al. 2015). The current study was conducted to determine the structure and function of defensin genes in lentil plant and to find similarities and differences between lentil defensins and other defensins. For the first time in this study, six members of the defensin gene family were identified and characterized in lentil. Furthermore, certain features such as signal peptides, functional domains, disulfide bridges, intron–exon boundaries, subcellular localization and antimicrobial activity, were predicted using in silico tools.

Materials and methods

Plant materials and growth condition

Seeds of lentil plant (Gachsaran cultivar) were potted in soil mixture consisting of peats and clay (50:50) and then transferred to greenhouse. Pots were kept at greenhouse standard photoperiod and temperature and were fertilized with NPK fertilizer (20:20:20) for 10 days. The seedlings (10 days) were used for DNA and RNA extraction.

DNA, RNA extraction and cDNA synthesis

Ten days seedlings were ground in liquid nitrogen. Genomic DNA was extracted using CTAB method (Doyle 1991). Total RNA was extracted using RNxplus RNA extraction buffer (SinaClon BioScience, Tehran, Iran) according to manufacturer's instructions. The quantity and quality of the extracted nucleic acids were determined using nanodrop and agarose gel electrophoresis. Genomic DNA was removed from total RNAs using DNase I (Thermo Fisher Scientific, Lenexa, USA). First-strand cDNA synthesis was performed on 2 μ g of total RNA using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Lenexa, USA). The successful synthesis of cDNA was confirmed by RT-PCR using specific elongation factor 1 alpha (*EF1a*) primers (Table 1).

Identification of defensin genes coding sequences

To identify coding sequences of defensin genes, all defensin coding sequences of dicotyledon plants (1955 sequences) were retrieved from the NCBI GenBank. The retrieved sequences were aligned against *L. culinaris* EST library (9539 ESTs) and the resulting sequences were pooled and assembled in Vector NTI 10.3 software (Invitrogen,

Table 1 Primers used for gene isolation

Primer name	Sequence (5'-3')	T_a (°C)
D1-F	AGAAATCAGTAGGTGTCTTGTC	52.9
D1-R	TATATTATGCGTCTTGTATTGGAG	
D2-F	CATGGAGAAGAAAACAGTAGC	53.3
D2-R	TTGCGTGTGGAGAAAGG	
D3-F	TGGATTATGGGGAGGAAAAC	54.3
D3-R	GCATGTCCATTGTTGAACAAAC	
D4-F	CTCGTTCAATTCTTTGGTTTCC	54.3
D4-R	CTTTCATCAACAATGTGTAGTGC	
D5-F	ATATTTGAGCCATTATGGAGAGG-3'	54.8
D5-R	GAACATAACAAAGACACATACAGC	
D6-F	TGGCTCGTTCAATTCCTATG	53.5
D6-R	ATTAACAATGTGTAGTACAGAAAC	
Ef1a-F	GCAGGTATGGTTAAGATGGTTCC	54.3
EF1a-R	TCTCCACACTCTTGATGACTCC	

Carlsbad, USA). The assembled (contigs) and non-assembled (singletons) sequences were aligned against GenBank nr database using BLASTn tool. The contigs and singletons were further analyzed for presence of complete defensin ORF. The contigs and singletons with the complete defensin ORF were used to design primer. Primer designing was performed using Allele ID 7.0 (Premier Biosoft, Palo Alto, USA) (Table 1).

Amplification and cloning of defensin cDNAs and genes

For amplification of defensin cDNAs and genes, PCR was performed using *Taq* DNA polymerase (SinaClon BioScience, Tehran, Iran) and gene specific primers (Table 1). Lentil genomic DNA and cDNA were used as template of PCR. After amplification, cDNA and gene sequences were T/A cloned into pTZ57R/T plasmid (Thermo Fisher Scientific, Lenexa, USA) and sequenced by Bioneer Company (Daejeon, Korea). The results of sequencing were analyzed by Vector NTI 10.3 (Invitrogen, Carlsbad, USA) and compared to GenBank using BLASTn tool.

Protein sequence analysis

Protein features, such as length, amino acid frequency, molecular weight, isoelectric point (pI), aliphatic index and grand average of hydropathicity (GRAVY) were predicted by CLC Main Workbench 5.5 (CLC Bio, Qiagen, Aarhus, Denmark) and ExPASy ProtParam tool (Gasteiger et al. 2005). Protein secondary structures were predicted by CLC Main Workbench 5.5 and PSIPRED server (McGuffin et al. 2000). Protein subcellular localization was determined using CELLO2GO tool (Yu et al. 2014). Protein functional

domains were predicted by Pfam, Interproscan and CDD servers (Marchler-Bauer et al. 2014; Finn et al. 2016; Jones et al. 2014).

Multiple sequence alignment and phylogenetic analysis

The multiple sequence alignment was performed using CLC Main Workbench 5.5 and Vector NTI 10.3. The phylogenetic analysis of isolated defensins was done by MEGA 6.0 (Tamura et al. 2013). The phylogenetic tree was constructed using the maximum likelihood method and evaluated by bootstrap test (1000 replications).

Protein interaction network

Due to lack of availability of lentil protein interactome, protein interaction network of isolated defensins was constructed using *Arabidopsis thaliana* interactome. The search for interaction partners was done by STRING 10.5 database, which is a database of known and predicted protein–protein interactions with direct (physical) and indirect (functional) associations (Szklarczyk et al. 2014).

In silico antimicrobial activity prediction

Antimicrobial activity of isolated defensins was predicted by CAMP (Collection of Anti-Microbial Peptides) server. In the CAMP server, antimicrobial activity of desired proteins is predicted by Support Vector Machine (SVM), Random Forest (RF), Artificial Neural Network (ANN) and Discriminant Analysis (DA) algorithms (Waghu et al. 2014).

In silico gene expression analysis

Fifteen RNA-seq libraries including leaf (SRX2334831), stem (SRX2334830), flower (SRX2334829), seed (SRX2334828), root (SRX2334827), pod (SRX2334826), immature pod (SRX2334825), flower bud (SRX3702581), seedling shoot (SRX3702578), etiolated seedling (SRX3702577), whole seedling (SRX3702567), root nodules (SRX3702565) and hydroponic roots (SRX3702569) were retrieved from NCBI SRA database. In addition, to in silico expression analysis of lentil defensins under fungi and wounding treatments, eighteen RNA-seq libraries (Table S1) including fungal treated and control leaves, as well as, two larval wounding (SRX2322960 and SRX2322961) libraries were downloaded from NCBI SRA database. The short-reads of mentioned libraries were mapped to the isolated defensins using CLC Main Workbench 5.5 and the RPKM values were calculated for all defensins.

Results

Characterization of defensin genes structure

Six contigs containing defensin complete ORF were identified from assembly of lentil EST library. RT-PCR confirmed the assembly results and showed fragments ranging in size from 225 to 260 bp. The cDNA sequences contained complete ORFs ranging in size from 222 to 228 bp. All ORFs started with ATG codon and terminated with TAA, TAG and TGA stop codons (Figs. S1–S6). The isolated cDNA sequences were submitted to GenBank with the accession numbers MF325783.1 (Defensin 1), MF325784.1 (Defensin 2), MF325785.1 (Defensin 3), MF325786.1 (Defensin 4), MF325787.1 (Defensin 5) and MF325788.1 (Defensin 6). Results of PCR on genomic DNA showed fragments with length of 315 to 705 bp (Figs. S7–S12). The full-length gene sequences contained introns ranging in size from 67 to 480 bp (Figs. S7–S12). All defensin ORFs were interrupted by only one intron. The gene names, cDNA length, DNA length, ORF length, intron length, number of introns and intron type have been summarized in Table 2.

Translation of the defensin ORFs produced peptides with length of 73 to 75 amino acids that had molecular weight between 8.1 and 8.4 kDa. The theoretical pI of these peptides varied from 6 to 9.41. The instability index of defensin peptides was predicted between 24.05 and 53.43. The aliphatic index of these peptides was between 59.87 and 79.05, whereas theoretical GRAVY was predicted between −0.236 and 0.113. In the amino

acid frequency comparison, Cys, Leu, Thr, Arg, Phe, Ser, Lys, Gly and Val showed the most frequency and Pro, Trp, and Tyr had the least frequency in defensin peptides (Fig. S13). The peptide name, peptide length, molecular weight (MW), isoelectric pH (pI), instability index, aliphatic index and grand average of hydropathy (GRAVY) have been shown in Table 3.

Prediction of subcellular localization showed that all defensin peptides are localized in the extracellular space and they contain signal sequences ranging in size from 24 to 28 amino acids. The gene ontology analysis showed that defensins are involved in response to biotic stimulus, killing of cells of other organisms and defense response to fungus. Protein secondary structure prediction revealed that alpha helix and beta strand structures were present in all defensins (Fig. S14). Functional domain search showed the presence of Knot1 domain in all defensins. The signal sequence site, subcellular localization site, secondary structures and functional domain have been indicated in Table 4.

Phylogenetic analysis

To understand the evolutionary distances among the defensin genes, unrooted phylogenetic tree was constructed using protein sequences from two model plants (*A. thaliana* and *Medicago truncatula*) species. The results showed all of the isolated defensins belonged to the gamma-thionin superfamily. The isolated defensins along with other defensins were classified into five subfamilies and different subgroups (Fig. 1).

All lentil defensins were categorized into two of the five subfamilies. Also, in these two subfamilies, lentil defensins

Table 2 Gene features of isolated defensin peptides

Name	cDNA length (bp)	DNA length (bp)	ORF length (bp)	Intron length (bp)	No. of introns	Intron type
Defensin 1	260	347	222	87	1	GT-AG
Defensin 2	248	335	225	87	1	GT-AG
Defensin 3	248	315	225	67	1	GT-AG
Defensin 4	230	336	228	106	1	GT-AG
Defensin 5	242	328	225	86	1	GT-AG
Defensin 6	225	705	225	480	1	GT-AG

Table 3 Physicochemical features of isolated defensin peptides

Name	Length (aa)	MW (kDa)	pI	Instability index	Aliphatic index	GRAVY
Defensin 1	73	8.16242	6	24.05	72.05	0.045
Defensin 2	74	8.40074	7.53	20.89	69.86	−0.236
Defensin 3	74	8.2007	9.41	53.43	67.3	0.111
Defensin 4	75	8.26753	8.52	34.73	59.87	0.113
Defensin 5	74	8.22787	9.32	28.64	79.05	0.034
Defensin 6	74	8.28175	9.14	49.32	63.24	0.092

Table 4 Functional characterization of isolated defensin peptides

Name	Signal sequence site	Localization site	Secondary structures	Functional domain
Defensin 1	1–27	Extracellular	2 α and 3 β	Knot1/Gamma-thionin
Defensin 2	1–27	Extracellular	3 α and 2 β	Knot1/Gamma-thionin
Defensin 3	1–24	Extracellular	1 α and 5 β	Knot1/Gamma-thionin
Defensin 4	1–28	Extracellular	1 α and 3 β	Knot1/Gamma-thionin
Defensin 5	1–24	Extracellular	2 α and 3 β	Knot1/Gamma-thionin
Defensin 6	1–27	Extracellular	1 α and 4 β	Knot1/Gamma-thionin

Fig. 1 Phylogenetic analysis of lentil defensins. Five subfamilies have been depicted by red, green, purple, yellow and black colors. The defensins of *L. culinaris*, *A. thaliana* and *M. truncatula* have been shown by filled circle, filled triangle and filled square, respectively. Numbers at each branch indicate bootstrap values

were classified into three subgroups. The defensins 1 and 2 were categorized together in one subfamily whereas defensins 3, 4, 5 and 6 were classified together in another subfamily (Fig. 1).

In multiple sequence alignment, the nucleotide sequences showed identity between 44 and 78% whereas protein sequences had similarity between 25 and 82% (Fig. 2 and Table S2). The Knot1 functional domain was partially conserved among lentil defensins, whereas the signal sequences were highly variable. Multiple sequence alignment also exhibited eight conserved Cys that formed four disulfide bridges. Furthermore, two Gly and one Asp residues were

found conserved in all lentil defensins. The signal sequences, Knot1 domain, conserved amino acids and disulfide bridges have been shown in Fig. 2.

Protein interaction network

The protein interaction network of six lentil defensins was composed of 16 nodes and 36 edges (Fig. 3). The edge scores were determined from 0.428 to 0.987. The interaction network was composed of functional groups, including enzyme inhibitors, pectinesterases, methylesterases, ubiquitin hydrolases, F-box proteins, and transcription factors. The defensins 1–5 showed interaction with 2, 5, 2, 6 and 6 proteins, respectively, whereas defensin 6 had no interaction partner. Defensins 1 and 3 only interacted with defensin peptides, whereas defensins 2, 4 and 5 had other interaction partners. All proteins in the network were directly or indirectly involved in defense responses. Predicted interaction network has been shown in Fig. 3.

In silico antimicrobial analysis

The results of analysis using CAMP server showed antimicrobial activity in all lentil defensins (Table 5). The SVM algorithm predicted antimicrobial values between 0.739 and 0.985. In the RF algorithm, antimicrobial values were determined from 0.703 to 0.9665. The antimicrobial values from 0.523 to 1 were computed by DA algorithm. The antimicrobial activity of all lentil defensins was confirmed by ANN algorithm.

In silico gene expression analysis

In silico gene expression analysis showed that defensins are differentially expressed in various tissues of lentil. In general, defensin 4 showed the highest expression level and defensin 6 displayed the lowest expression level among all tissues. Defensin 1 exhibited the highest expression level in the root tissue and the lowest expression level in the seedling shoot. Defensin 2 was highly expressed in the root tissue. Defensins 3 and 5 showed the highest expression level in the root, immature seed, and pod tissues. Defensin 4 was

Fig. 2 Multiple sequence alignment of lentil defensins. Signal sequence, Knot1 domain and disulfide bridges have been shown

Fig. 3 Protein–protein interaction network of defensins. The Nodes (colored circles) are defensins and their interaction partners. The Edges (colored lines) represent protein–protein interaction among the nodes

highly expressed in all examined tissues. Defensin 6 showed the highest expression level in the root, leaf, and etiolated seedling tissues (Fig. 4).

The results also showed that defensins are differentially expressed in response to *A. lentis* inoculation (Fig. 5). Only defensins 1, 4 and 6 responded to fungal treatment and the other defensins did not show any expression. Expression of

defensin 1 was significantly increased 6 h after fungal inoculation. Defensin 4 was significantly down-regulated between 2 and 6 h post-inoculation. Defensin 6 was significantly up-regulated between 2 and 6 h post-inoculation (Fig. 5).

In wounding treatment, defensins were differentially expressed and only defensins 1, 2 and 4 responded to treatment. Defensins 1 and 2 were significantly up-regulated

Table 5 Predicted antimicrobial values of defensins

Name	ANN algorithm	DA algorithm	RF algorithm	SVM algorithm
Defensin 1	☑	0.523	0.703	0.751
Defensin 2	☑	0.788	0.9665	0.739
Defensin 3	☑	1	0.943	0.978
Defensin 4	☑	0.814	0.8515	0.857
Defensin 5	☑	0.998	0.8335	0.985
Defensin 6	☑	0.985	0.776	0.937

The SVM, RF, ANN and DA are prediction algorithm. Values ≥ 0.5 = Antimicrobial activity

☑ Antimicrobial activity confirmation

Fig. 4 Heat map of tissue-specific expression pattern of *L. culinaris* defensins. The range of RPKM values is from 0 (red) to 2000 (white) to enhance visualization

in response to wounding treatment. Defensin 4 showed a different trend and was significantly down-regulated in response to wounding treatment (Fig. 6).

Discussion

Defensins are the major component of the plant immune system. They play multiple roles in response to stresses and during growth and development (Cools et al. 2017; Lacerda et al. 2014; Vriens et al. 2014; Van der Weerden and Anderson 2013). Until now, defensins have been investigated in some monocot and dicot plants. Due to lack of a reference genome for lentil, it is difficult to study the defensin gene family in this plant.

In the current study, we have applied experimental and *in silico* methods to identify and classify defensins in *L. culinaris*. The six cDNAs and six DNAs corresponding to lentil defensins were identified and cloned from lentil transcriptome and genome (Figs. S1–S12), thus providing the first report of the defensin gene family in lentil. Although, there is one report of identification of defensin peptide in lentil (Finkina et al. 2008). The precise size of the defensin gene family in lentil will be known only when the reference genome becomes available for this plant.

Transcriptome analysis of lentil showed the presence of six defensin genes. Based on the phylogenetic analysis, the isolated defensin genes were assigned to two subfamilies (Fig. 1). Structural and functional study of plant defensins have shown that their family is classified into four subfamilies (Finkina et al. 2008). Subfamily I includes antibacterial defensins and antifungal defensins that cause hyphal distortion. Members of subfamily II show antifungal activity through morphological effects. Subfamily III includes antibacterial and insecticidal plant defensins. Members of subfamily IV display both antibacterial and antifungal activity (Finkina et al. 2008). The belonging of isolated defensins to only two subfamilies may be due to the presence of more members in this gene family in lentil plant.

Gene structure analysis of lentil defensins showed two exons and one intron in their structure (Figs. S1–S12). The introns are located within the signal peptides in all lentil defensins. The lentil introns vary in length, whereas exons size is fairly conserved. Defensin genes share a typical exon-intron-exon structure, although exceptions have been previously reported (Houlne et al. 1998; Giacomelli et al. 2012). Due to the existence of only one intron in defensins gene structure, alternative splicing do not occur in these genes (Black 2000). Our results are consistent with previous studies showing that most of the plant defensins contain single intron (Spelbrink et al. 2004; Hanks et al. 2005).

Subcellular localization analysis showed secretory signal peptide in all lentil defensins (Fig. 2). The secretory signal peptides help defensins to localize in extracellular space where they show their biological activity (Cools et al. 2017). According to the pre-protein structure, plant

Fig. 5 Expression pattern of defensins 1, 4 and 6 under fungal treatment

Fig. 6 Expression pattern of defensins 1, 2 and 4 under wounding treatment

defensins are classified into two classes. The first class contains defensins with a signal peptide and a mature defensin peptide. The signal peptide targets the defensin to the endoplasmic reticulum and then to the secretory pathway. The second and less common defensin group comprises defensins with an additional C-terminal pro-domain that is removed subsequently (Vriens et al. 2014). Pathogens invade the host cells from extracellular space. Therefore, the presence of defensins and other AMPs in extracellular space is necessary to combat with pathogens. Our results are consistent with previous studies showing that most of the plant defensins have secretory signal peptide in their structure (Lay et al. 2003; Oomen et al. 2011).

Multiple sequence alignment showed eight conserved Cys amino acids in all lentil defensins (Fig. 2). Conserved Cys residues in the lentil defensins are necessary for disulfide bridges formation. Intramolecular disulfide bridges stabilize defensins structure and are required for proper biological activity (Agrawal et al. 2017; Van der Weerden and Anderson 2013). Conserved Gly residues form γ -core motif in the lentil defensins (Fig. 2). The γ -core motif (GXCX₃₋₉C) is a group of positively charged amino acid residues, which is vital for antimicrobial activity. The γ -core motifs are present in the disulfide bridge containing antimicrobial peptides (Van der Weerden and Anderson 2013; Lacerda et al. 2014).

The alpha helices and beta strands are vary in number and position in lentil defensins (Fig. S14). This variation in secondary structures may correlate with different biological

roles of plant defensins such as antibacterial activity, anti-fungal activity and enzyme inhibitory activity (Van der Weerden and Anderson 2013). The presence of conserved Cys residues, disulfide bridges, γ -core motif and specific secondary structures correlated with antimicrobial activity in lentil defensins.

Like other proteins in the biological systems, the lentil defensins act through the protein interaction network (Fig. 3). Defensins 4 and 5 interacted with invertase/pectin methylesterase inhibitor enzyme in their protein interaction network. The invertase/pectin methylesterase inhibitors play an important role in osmotic stress, senescence, wounding, the plant defense processes, cell wall integrity and seed development (An et al. 2008; Lionetti et al. 2017). In *(A) thaliana*, two invertase/pectin methylesterase inhibitors protect cell wall integrity during *(B) cinerea* infection (Lionetti et al. 2017). Our results showed that only lentil defensin 4 probably interacts with invertase/pectin methylesterase inhibitors during *A. lentis* infection. Defensins 4 and 5 also showed interaction with ubiquitin carboxyl-terminal hydrolase (Fig. 3). The ubiquitin carboxyl-terminal hydrolase enzymes have a key role in the jasmonic-acid mediated signaling pathway, protein de-ubiquitination, and ubiquitin-dependent protein catabolic process (Bachmair et al. 2001). These proteins may interact with defensins through jasmonic-acid dependent defense pathways. Both ubiquitin carboxyl-terminal hydrolase and defensin were up-regulated in *Rubus occidentalis*

under *V. dahliae* infection (Bushakra et al. 2015). Our results showed similar trend for lentil defensin 4 under *A. lentis* infection. Defensin 4 probably interacts with ubiquitin carboxyl-terminal hydrolase through jasmonic-acid mediated signaling pathway. Defensin 2 showed interaction with myb-like proteins and F-box proteins (Fig. 3). MYB transcription factors play important regulatory roles in numerous plant biological processes such as developmental processes and defense responses (Yanhui et al. 2006). F-box proteins are involved in many plant processes including growth, development, and defense processes. The F-box protein *ACRE189/ACIF1* showed an important role in cell death and defense process in the tobacco and tomato plants under pathogen treatment (van den Burg et al. 2008). The F-box protein *COII* played important role in jasmonate-regulated defense responses in *A. thaliana* (Xu et al. 2002; Jain et al. 2007). Our results revealed that lentil defensin 2 may interact with MYB and F-box proteins in wounding stress.

The defensin genes were differentially expressed in the lentil tissues but seemed to be preferentially expressed in the seed, pod and root tissues where cells are faced with pathogens (Fig. 4). The defensins are more abundantly expressed in immature seed, pod, and root than in other tissues, showing that they protect these vital plant tissues from pathogens invasion. A different trend of defensins expression was observed in the hydroponic roots compared to roots grown in soil. This difference is probably due to different growth condition. The high expression of defensin 4 in all evaluated tissues showed that this gene has additional non-defensive roles during normal plant development. So far, different roles (from antimicrobial activity to heavy metal tolerance) have been reported for plant defensins and plant defensins structure may provide a framework for display of numerous activities (Van der Weerden and Anderson 2013).

Of the six isolated defensins, three were induced by wounding and fungal treatments. Defensins 1 and 4 responded to both fungal and wounding treatments. Defensin 2 only up-regulated in wounding treatment, whereas defensin 6 only responded to the fungal treatment. Defensin 1 showed a common response to fungal and wounding treatments and up-regulated in both treatments. Defensin 4 was down-regulated in response to fungal and wounding treatments. The expression profiles of lentil defensins were consistent with previous reports that showed up-regulated and down-regulated genes in *(A) thaliana* infected with *Alternaria brassicicola* (Thomma and Broekaert 1998). Similar trend was also observed in *(B) cinerea* infected grapevine (*Vitis vinifera*) and infection led to up-regulation or down-regulation of defensin genes (Giacomelli et al. 2012). Resistance against necrotrophic fungal pathogens requires jasmonic-acid mediated pathway, leading to the activation of defense proteins including defensins (Tsfaye et al. 2013).

Members of defensin gene family have different role in plants. Some of those are multi-functional and play role in growth and development and response to different stresses, whereas some others are single-functional and are involved only in a specific process (Do et al. 2004; Hanks et al. 2005; Meyer et al. 1996; Van der Weerden and Anderson 2013; Vriens et al. 2014).

The reason for this difference in function is probably due to the difference in the expression pattern and distribution of particular cis-acting elements in their promoters (Siddique et al. 2011; Manners et al. 1998; Thomma and Broekaert 1998; Do et al. 2004; Meyer et al. 1996; Van der Weerden and Anderson 2013). Multi-functional defensins have simultaneously different cis-acting response elements in their promoters, whereas single-functional defensins may only contain specific cis-acting response elements (Manners et al. 1998; Siddique et al. 2011).

Conclusion

The current study is the first report of the isolation and characterization of six members of the defensin gene family in lentil. In our study, a comprehensive analysis including gene structure analysis, functional domains prediction, phylogenetic analysis, protein features analysis, antimicrobial analysis, interaction analysis and gene expression analysis was performed on lentil defensin gene family. The results of this study showed that lentil defensins, like other plant defensins, contain only one intron and produce small peptides. Presence of signal peptide, extracellular localization, the presence of Knot1 functional domain, conserved Cys amino acids and disulfide bridges, interaction with defense and signaling proteins, antimicrobial activity and spatiotemporal expression are the other features of these peptides. The comprehensive information found in the current study expands our information about the involvement of defensin genes in plant growth and development and in response to biotic and abiotic stresses. However, the additional members of defensin gene family should be identified and characterized in lentil. Moreover, the role of defensins in response to stress, especially in response to abiotic stress should be further analyzed.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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