Identification and Characterization of Defensin Genes in *Brassica juncea* and *Camelina sativa* and Analysis of their Expression in Response to Biotic and Abiotic Stress

Gamage Dona Gaya Chaturani, Zahoor Ahmad Mir, Prashant Mishra and Anita Grover*

National Institute for Plant Biotechnology, New Delhi, India

*Corresponding Author: Anita Grover, National Institute for Plant Biotechnology, New Delhi, India

Received: October 28, 2019; Published: November 29, 2019

DOI: 10.31080/ASAG.2019.03.0732

Abstract

Plants are attacked by various biotic and abiotic factors throughout their lives and how efficient and effective the defense mechanisms are, determine the survival. Defensins, belong to Pathogen Related (PR) protein class-12. They are one of the key modulators in plant defense mechanisms which have diverse functions including inhibitory effect on a broad range of phytopathogenic fungi, and are involved in biotic stress response. In depth study on defensins gives an insight into their role in defense which further can be used in conferring disease resistance in crop plants. *In-silico* analysis identified 56 putative defensins in *C. sativa* (resistant to *Alternaria* blight) whereas, only six were identified in *B. juncea* (susceptible to *Alternaria* blight). Multiple sequence alignment and consensus analysis confirmed the conserved eight cysteine residues, and structural analysis revealed the presence of an α -helix and triple strand anti parallel β -sheets. Defensin genes identified were having a single intron, and identification of Methyl Jasmonate (MeJA) responsive elements in promoter analysis confirm their regulation under Jasmonic Acid (JA) signalling pathway. These defensins were phylogenetically related to other *Brassica* species as well as to *Arabidopsis* indicating their close evolutionary relationship. Expression analysis indicated that defensin is responsive to JA, Salicylic Acid (SA), *Alternaria* infection, and wounding. The antagonistic effect between SA and JA was observed in defensin gene expression in response to their exogenous application. This study will provide an important foundation for further investigation on defensin as a possible source for creating disease resistance in transgenics.

Keywords: Defensin; Brassica; Camelina; JA; SA; *Alternaria* Blight

Introduction

Alternaria blight of cruciferous vegetables, incited by different species of *Alternaria*, remains an increasing threat to Brassicaceae crops throughout the world. In oil seed rape, *A. brassicae* is the dominant invasive species [37]. *A. brassicae*, is one of the most common and destructive disease of Indian mustard (*Brassica juncea*) and the yield losses have been estimated to range from 35 to 46% [32]. Conventional breeding to develop resistant cultivars in *B. juncea* against *A. brassicae* is confounded due to non-availability of suitable resistant sources within the available germplasm of cultivated species of Brassica [35], though some varieties differ in their resistance level. A high degree of resistance to *A. brassicae* has been found in the wild relatives of Brassica outside the tribe Brassicaceae including *Camelina sativa* [21]. Thereby, there are

necessities of adopting biotechnological strategies to improve resistance against this pathogen. Biotechnological approaches can successfully be utilized to develop resistant variety of *B. juncea* provided that molecular mechanism of defense is delineated.

The molecular mechanisms underlying activation of plant defense responses are exceedingly complex [14] and understanding how plants defend themselves against a range of pathogens is therefore of crucial importance for successful agriculture [1]. Once plant defense responses are activated at the site of infection, a systemic defense is often triggered in distal plant parts to protect these undamaged tissues against subsequent invasion by the pathogen [40]. Systemic defense is controlled by a complex signal transduction network [44] that coordinate the overall defense responses of the plant [19]. Resistance to biotrophic pathogens is

mediated through phyto-hormones such as salicylic acid (SA) [40] and necrotrophic pathogens by jasmonic acid (JA) and ethylene (ET)-signaling pathways [28].

Various novel proteins are induced [29] collectively known as PR proteins [09] is a group of the most important inducible defense-related antifungal proteins [33]. They are associated with the development of systemic acquired resistance in wide range from cell wall rigidification to signal transduction and antimicrobial activity [26]. They are the key modulators of plant defense [13] which cause activation of a different set of defence related genes, including plant defensin genes [44]. Plant defensins which belong to PR-12 family of PR proteins, are cysteine rich, highly basic [50] prominent cationic peptide in plants [03] with different roles in defense [25, 39]. They are one of the largest families of antifungal peptide molecules [10]. Since the beginning of 1990s, many cationic plant cysteine rich antimicrobial peptides have been studied and plant defensins were first described in the seeds of wheat and barley [05, 31].

The main biological function of plant defensins was found to inhibit the growth of broad range of phyto-pathogenic fungi at micro-molecular concentrations. The precise mechanism of action that is employed by plant defensins to inhibit the fungi is not completely understood, although it is generally accepted that they act at the level of the plasma membrane [23]. While some results point to cellular membrane as the point of action, others suggest intracellular targets [49]. There are two major hypothesis which explain the mechanism of action of antimicrobial defensin: the carpet model and the pore model. In both models, defensins are described to interact with the negatively charged molecules present at the cell membrane of pathogens, causing an increase of its permeabilization, leading to cell leakage and death by necrosis [22]. In addition to being antimicrobial, plant defensins are also involved in the biotic stress response, as well as plant growth and development [48].

In Brassica only a few defence related genes have been reported. These include PR proteins including few plant defensins [15], β -glucanases [36], and Chitinases [55]. Tiwari [53] studied the effect of methyl jasmonate on disease severity and expression of plant defensin gene during *A. brassicae* infection in *Arabidopsis* and he has reported that transcripts of *PDF1.2* accumulated at a greater level upon challenge inoculation with *A. brassicae* along with jasmonic acid compared to treatment containing pathogen as well as jasmonic acid alone. However no attempts have been undertaken to find the number of defensin genes present in *B. juncea* or in *Alternaria* resistant *C. sativa* and the structural and functional

characteristics of defensins from above two genotypes remain to be unclear. Rawat [41] isolated the full-length pathogen-inducible plant defensin gene from *B. juncea* and have reported its similarity with gamma thionin and knottin families of plant antimicrobial peptides. Efforts are being made in order to have sufficient knowledge about the genes induced during infection and their regulation measures. Therefore the objective of this study is to characterize different defensin genes in *B. juncea*, *C. sativa* and related species to find their structure, evolution, cellular localization, and studying their regulation in response to SA, JA and *Alternaria* infection.

Materials and Methods

Genome wide identification of defensin genes in *B. juncea* and *C. sativa*

The available *Arabidopsis thaliana* defensin genes were downloaded from TAIR database (<https://www.arabidopsis.org/>), and their coding sequences were identified using ORFfinder program (<https://www.ncbi.nlm.nih.gov/orffinder/>). By using the ExPASy translate tool (<https://web.expasy.org/translate/>) the coding sequences were translated to peptide sequences. The above peptide sequences of *A. thaliana* defensin genes were queried against *B. juncea* and *C. sativa* peptide sequences in BRAD database (<http://brassicadb.org/brad/>) to find putative defensins in *B. juncea* genome using blastp. From the blast output the peptide sequences showing >100 bit score were selected and redundant hits were removed to select unique sequences.

Protein structure, conserved domain identification and computation of physical and chemical parameters

The NCBI-CDD database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) was used to analyze the conserved domain of all non-redundant sequence and SMART (<http://smart.embl-heidelberg.de/>) and Pfam (<https://pfam.xfam.org/>) were used to confirm that identified genes were members of defensin family. Those protein containing gamma-thionin domain under Knot1 superfamily were defined to be belonging to the defensin family (previously known as gamma-thionin family). Full length amino acid sequences of the selected defensin proteins were compiled and aligned using ClustalW and the alignment was visualized by Bio Edit V 7.0.4. software to find conserved regions, and the three dimensional secondary structure of defensin proteins were constructed by a ProMod3 Version 1.3.0. homology modelling method using SWISS-MODEL server (<https://swissmodel.expasy.org/interactive>) to confirm that the selected proteins have structures similar to plant defensins, as they are generally defined by their conserved cysteine scaffold with α -helix and triple strand anti parallel β -sheets connected to the scaffold. Computer analysis of the amino acid sequence to com-

pute chemical and physical parameters were performed with the ProtParam tool on the ExPASy server (<https://web.expasy.org/protparam/>) and possible disulphide bridges were determined using the DISUFIND server (<http://disulfind.dsi.unifi.it/>). The subcellular localization of each defensin protein of *B. juncea* and *C. sativa* was predicted using Plant-mPLoc (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>).

Gene Structure construction and prediction of *cis*-acting elements

The defensin sequences retrieved from BRAD database were employed to identify respective genes from *B. juncea* and *C. sativa* genome using local blast in Bio Edit V 7.0.4 software. The exon-intron structure of *B. juncea* defensin encoding genes were determined based on alignment of coding sequences with corresponding genome sequences and graphical display was created using online Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). Conserved *cis*-acting regulatory elements in the promoter region of the putative defensin genes were analyzed by plant care database (<http://bioinformatics.psb.ugent.be/webtools/plant-care/html/>).

Phylogenetic analysis of defensin proteins of *B. juncea*, *C. sativa* and related species

To compare the composition of the defensin gene family in Brassica, defensin genes were identified in three other Brassica species, *B. rapa*, *B. napus* and *B. oleracea* and also the wild type *C. sativa* (<http://brassicadb.org/brad/>) using the same method. To study the evolutionary relationships among *B. juncea*, *B. rapa*, *B. oleracea*, *B. napus* and *A. thaliana* defensins, an unrooted neighbor-joining phylogenetic tree was constructed using MEGA 7.0 software based on the amino acid sequences of the defensin proteins of above genotype and a bootstrap test with 1000 replicates was performed.

Expression analysis of defensin genes in *B. juncea* and *C. sativa* in response to SA, JA and *A. brassicae* infection and wounding

B. juncea and *C. sativa* plants were raised from seeds in pots and were maintained at 25 °C for 16 h light/8 h dark in glass house at National Phytotron Facility, Indian Agriculture Research Institute (IARI). *A. brassicae* isolated from disease infected field grown plants was cultured on Radish Dextrose Agar medium and was identified by Indian Type Culture Collection, IARI (ID No. 81651). Conidial suspensions were prepared by scraping sporulated mycelium from 21-day old cultures and suspending in sterilized distilled water. Conidial concentration was adjusted to 5×10^3 conidia/ml using hemocytometer. 45 days old healthy plants with six leaves,

were treated with conidial suspensions using a hypodermic needle and control plants were treated same way with sterilized distilled water. To study the effect of hormone on defensin gene expression, plants were sprayed with 100 μ M MeJA, and 2 mM SA solutions separately and control plants were sprayed with sterilized distilled water. Small puncture wounds were made on the leaves using a hypodermic needle filled with sterilized distilled water, to analyze the effect of wounding on the expression of defensin genes. Leaf samples were collected at 3, 6, 12, 24, 48, 72 and 96 h post treatment. Total RNA was isolated from leaf tissues using TRIzol reagent (Invitrogen™) and first strand cDNA synthesis was performed from 2 μ g total RNA using Superscript III cDNA synthesis kit (Invitrogen™) following the manufacturer's instructions. Quantitative real time PCR was performed using SYBR Premix Ex Taq Kit (Takara™) run at 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s with F-5' CATGAAGCTCTCTATGCG 3' and R-5' CGATGGATCAGCGATTCTTCTGG 3' primers. *β -tubulin* was used as the reference gene for mRNA relative expression pattern analysis. The reactions were performed with three biological replicates and three technical replicates per sample. The relative quantification method ($\Delta\Delta$ CT) was used to evaluate quantitative variation between the samples.

Results and Discussion

Genome wide identification of defensin genes in *B. juncea* and *C. sativa*

During *in-silico* analysis, fifteen defensin protein coding sequences were identified in *A. thaliana* from the TAIR database. They were sub divided into three sub families, which seven belonging to family I, six to family II and two plant defensins in family III. All the coding sequences were converted into their respective amino acid sequences and used as a reference to find the plant defensins in *B. juncea*, *B. rapa*, *B. oleracea*, *B. napus* and *C. sativa*. Availability of *B. juncea* genome sequence made it possible to identify all the defensin proteins present in Brassica. When *B. juncea* genome was queried against 15 defensin proteins of *A. thaliana* as described above, only six putative defensin proteins namely BjuB040113, BjuB040109, BjuB027448, BjuA030410, BjuA025766 and BjuB040111 were identified by the analysis that matches to the criteria of plant defensins. However comparative to low number of defensins in *B. juncea*, 56 defensin proteins were identified in *C. sativa* belonging to plant defensins. Twelve defensins were identified both in *B. rapa* and *B. oleracea*. Surprisingly no defensins were identified in *B. nigra*. Since *B. juncea* was evolved from cross between *B. nigra* and *B. rapa*, identification of only six defensins in *B. juncea* can be justified. However total 42 of defensin proteins were identified in *B. napus* indicating the possibility of gene duplications.

Analysis of physical, chemical and structural properties of defensin proteins

Computer analysis of physical and chemical parameters of all the defensin proteins was done by ProtParam (Table 1). The length of the defensin proteins ranged from 77 to 82 amino acid residues in *B. juncea* while in *C. sativa* it ranged from 72 to 131 amino acid residues. Relative molecular mass varied from 7.79 kDa to 14.33 kDa, while the theoretical *iso*-electric point measurement varied from 4.81 to 12.00. Instability index of all the defensin proteins identified was also calculated. A protein whose instability index is

smaller than 40 is predicted as stable; a value above 40 predicts that the protein may be unstable. Instability index indicates that four *B. juncea* defensin proteins out of six proteins were unstable while most defensins in *A. thaliana* and *C. sativa* were stable. Instability index provides an estimate of the stability of the protein in a test tube. Calculations on aliphatic index indicated that it varies with 32.52 to 91.17 in all three genotypes. Grand average of hydropathy (GRAVY) calculations indicated that most of the defensins are polar. GRAVY is an indication of hydropathy values of amino acids in a protein; negative values indicates that the protein is non-polar and positive values indicates that the protein is polar.

Prot. ID	Number of aa	Molecular Weight (kDa)	Theoretical pI	Instability index	Aliphatic index	Number of -ve residue	Number of +ve residue	GRAVY
NM_106233.4	80	8.709	8.47	27.49	74.50	4	7	0.339
NM_123809.4	80	8.518	8.14	27.68	81.88	4	6	0.454
NM_128161.2	80	8.640	8.14	28.23	78.12	4	6	0.389
NM_123810.3	80	8.550	8.14	25.27	75.75	4	6	0.358
NM_128160.3	80	8.580	8.14	25.28	78.25	4	6	0.436
NM_101817.4	78	8.840	8.44	55.88	52.69	5	8	0.113
NM_104375.2	80	9.139	5.65	31.07	65.88	9	8	0.006
NM_126272.4	77	8.056	9.02	28.58	91.17	5	8	0.600
NM_126271.3	77	8.524	9.37	40.13	63.25	3	10	0.201
NM_126273.4	77	8.544	9.63	39.18	59.48	3	12	0.004
NM_104788.4	76	8.289	8.52	51.47	80.79	5	8	0.182
NM_125761.2	73	8.387	8.92	56.73	54.79	4	9	-0.130
NM_126274.3	73	7.718	8.92	76.05	73.56	3	8	0.281
NM_123194.3	122	13.122	6.05	31.71	67.95	12	11	-0.130
NM_119153.2	129	13.862	5.57	43.91	55.89	12	10	-0.154
BjuB040113	80	8.734	8.47	30.55	78.25	4	7	0.335
BjuB040109	80	8.862	9.14	29.92	68.50	5	12	0.101
BjuB027448	77	8.574	9.33	45.54	58.18	4	11	0.031
BjuA030410	78	8.814	8.85	57.46	60.13	4	9	0.103
BjuA025766	83	8.592	12.00	89.44	64.34	6	13	0.200
BjuB040111	82	8.905	6.24	63.66	68.05	8	7	0.170
Csa07g047380.1	80	8.734	8.47	26.02	74.50	4	7	0.225
Csa09g080580.1	80	8.691	8.47	26.00	75.75	4	7	0.291
Csa16g039940.1	80	8.744	8.47	27.48	74.50	4	7	0.215
Csa16g039930.1	80	8.619	8.15	31.90	77.00	4	6	0.310
Csa13g034620.1	80	8.665	8.15	29.77	75.67	4	6	0.351
Csa09g080550.1	80	8.707	8.16	32.96	77.01	4	6	0.348
Csa09g080530.1	80	8.676	8.48	28.94	73.38	4	7	0.201
Csa09g080540.1	80	8.709	8.15	31.90	75.74	4	6	0.324
Csa09g080520.1	80	8.736	8.48	28.94	73.99	4	7	0.246

Csa09g080500.1	80	8.799	8.48	29.13	75.01	4	6	0.309
Csa16g039910.1	80	8.798	8.47	29.11	75.77	3	6	0.308
Csa16g039920.1	80	8.742	8.87	25.59	68.50	4	7	0.241
Csa09g080480.1	80	8.792	8.87	22.87	78.00	3	8	0.143
Csa07g047330.1	80	8.790	8.14	23.93	80.50	3	8	0.179
Csa05g073650.1	80	8.846	6.00	24.05	64.75	5	4	0.325
Csa05g073660.1	80	8.831	6.00	24.05	69.62	5	4	0.416
Csa16g039870.1	80	8.806	8.87	23.93	85.38	3	8	0.246
Csa11g046460.1	79	8.782	8.42	33.48	65.57	4	7	0.161
Csa07g047360.1	75	8.195	8.36	28.85	86.00	4	6	0.281
Csa12g074210.1	78	8.693	8.42	33.78	71.41	4	7	0.238
Csa09g080510.1	80	8.792	8.16	33.11	60.00	4	6	0.233
Csa07g047340.1	82	9.064	8.16	35.51	80.98	5	7	0.580
Csa16g039900.1	82	8.873	8.93	30.00	73.90	3	8	0.556
Csa16g039890.1	82	8.961	8.49	33.50	94.02	5	8	0.657
Csa07g047350.1	82	9.022	8.72	27.38	86.95	5	9	0.570
Csa03g023400.1	78	8.844	8.45	55.04	51.41	5	8	0.047
Csa17g025410.1	78	8.695	8.12	45.86	51.12	5	7	0.131
Csa11g002160.1	78	8.934	8.77	35.63	65.13	5	10	-0.158
Csa04g007620.1	77	8.671	10.04	42.45	56.88	3	13	-0.044
Csa06g002180.1	77	8.721	10.00	42.55	58.88	3	13	-0.053
Csa06g002170.1	77	8.694	9.69	37.13	61.95	4	13	-0.023
Csa04g007610.1	77	8.720	9.49	41.86	61.90	4	12	0.006
Csa00456s080.1	112	12.583	9.77	41.80	63.48	8	19	-0.157
Csa16g052960.1	74	7.998	7.52	49.61	81.62	5	6	0.408
Csa00456s090.1	73	8.240	8.96	69.01	68.08	5	10	0.047
Csa18g037270.1	72	8.435	8.74	62.68	55.56	5	9	-0.133
Csa06g002200.1	73	7.792	8.92	54.62	73.56	3	8	0.281
Csa04g007630.1	73	7.802	8.92	59.89	73.56	3	8	0.270
Csa00456s050.1	73	7.823	8.91	54.62	73.50	3	8	0.255
Csa16g045530.1	122	12.937	8.26	29.81	58.36	8	12	-0.053
Csa10g044380.1	130	14.004	7.79	41.00	58.38	9	11	-0.060
Csa12g017170.1	131	13.978	8.09	36.48	57.33	8	11	-0.145
Csa11g013400.1	131	14.083	7.81	39.82	58.78	10	12	-0.147
Csa12g084420.1	120	12.842	7.31	57.62	61.67	10	11	-0.072
Csa20g039330.1	124	13.490	6.65	22.24	73.87	13	13	0.095
Csa12g017150.1	130	14.169	5.65	26.82	57.08	14	12	-0.204
Csa10g012480.1	130	14.335	6.67	33.24	36.77	14	14	-0.376
Csa11g073450.1	113	12.351	8.48	18.48	80.27	9	14	0.245
Csa11g013370.1	111	12.098	4.81	42.21	32.52	16	10	-0.601
Csa12g085440.1	114	12.231	8.28	56.83	66.67	5	8	-0.112
Csa17g089850.1	123	13.264	5.21	55.25	68.21	10	9	0.004

Table 1: Chemical and physical parameters of defensin proteins in *A. thaliana*, *B. juncea* and *C. sativa*.

Controlling the stability of cellular proteins is a fundamental way by which cells regulate growth, differentiation, survival, and development. Measuring the turnover rate of a protein is often the first step in assessing whether or not the function of a protein is regulated by proteolysis under specific physiological conditions [56]. The half-life is a prediction of the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. ProtParam estimates the half-life by looking at the N-terminal amino acid of the sequence under investigation [12] and it was estimated that defensin proteins have more than 10 hrs of half-life in *E.coli*.

The subcellular localization prediction revealed that these proteins exhibited cell membrane and vacuolar localization. According to Donnes and Hoglund [8], the organelles present unique biological conditions to the proteins. It has been observed that proteins from different organelles differ in their overall amino acid composition and each protein has evolved over time to function optimally in a certain subcellular localization. Plant defensins seem to have specific activities depending on whether they are over-expressed by plant tissues or applied externally [38]. The subcellular localization of plant defensins thus appears to be a crucial point to understand their dual action on plant cells. As all plant defensins have a strongly hydrophobic N-terminal sequence that may serve as a signal peptide and have no obvious internal retention signal, they are generally predicted to be secreted proteins. However, most of the *B. juncea* plant defensins are predicted to be localized in vacuole. The same was observed [24] in NaD1 in *Nicotiana glauca* flowers. However, their internal location is not incompatible with their antimicrobial activity as Sticher, *et al.* [46] confirmed that when cells are damaged, defensins are

released from internal stores to protect tissues against fungal pathogens.

The defensin proteins are characterized by specific sequence diversity and structural feature homology. The protein domains of these defensin proteins were identified and similar protein structure and domain compositions were found, demonstrating that the protein structure is remarkably conserved within the plant defensins. All non-redundant sequence were used to confirm that identified proteins of *B. juncea* were members of defensin family (Figure 1). Those proteins containing gamma-thionin domain under Knot1 superfamily were defined to be belonging to the defensin family [previously known as gamma-thionin family]. Defensin and defensin like peptides are functionally diverse namely disrupting microbial membranes and acting as ligands for cellular recognition and signalling. According to Stotz, *et al.* [47], the first members of the family of plant defensins were isolated from wheat and barley grains in the early 1990s and those proteins were originally called gamma-thionins because of their size and cysteine content that were found to be similar to thionins. Subsequently gamma-thionins homologous proteins were identified and it as noticed that the plant peptides' structural and functional properties resemble those of insect and mammalian defensins, and therefore later termed the family of peptides 'plant defensins' in. Gachomo, *et al.* [11] also reported that the plant defensins are structurally similar to their insect counterparts despite the low amino acid sequence similarity between these two organisms. Maarof and co-workers [30] also reported the presence of Knot1 domain and gamma-thionin domain in their studies on CDef1 in *Capsicum*. The Knot1 domain or knottins represent plant antimicrobial peptides, plant amylase inhibitors, plant gamma-thionins and arthropod defensin.

Figure 1: The presence of gamma-thionin domain under Knot1 superfamily in, *B. juncea* and *C. sativa* defensins.

In addition to the conserved domains, other conserved motifs could also be important for the function of plant defensins. Plant defensins share a common motif consisting of an α -helix and triple strand anti parallel β -sheets, a feature that is also shared by several toxins from insects, scorpions, honeybees and spider venom [11]. The structural features of above defensins were analyzed in order to understand the functional characteristics of defensin family. Similar to the domain distribution results, the modeled defensins also share similarity with each other in terms of secondary struc-

ture as all the defensin proteins were represented by an α -helix and triple strand anti parallel β -sheets (Figure 2). Despite their structural similarity, plant defensins have great diversity in amino acid sequence. This variation in the primary sequence is associated with the specificity and diverse biological activities of antimicrobial peptides such as antibacterial, antifungal, antioxidant and other various antimicrobial activities [23]. Kaewklom, *et al.* [18] reported that recently, a conserved Y-core motif composed of two antiparallel β -sheets and an interposed loop has been identified and shown to be important for their functions.

Figure 2: Secondary structure analysis of *B. juncea* and *C. sativa* defensins.

Multiple sequence alignment of *B. juncea* and *C. sativa* defensin proteins revealed a highly conserved eight cysteine residues near the N-terminal domain of the proteins (Figure 3 and Figure 4). This observation is consistent with the finding of Lay, *et al.* [23], who suggested that the conservation of these residues may be due to their roles in providing stability and in the folding mechanism, especially the cysteines involved in the formation of disulfide bri-

dges. The amino acid sequence conservation of defensin proteins is limited to a small number of residues. These conserved residues are absolutely restricted to the eight cysteines, a glycine residue, as well as a serine and an aromatic residue (F/W/H/Y) that is always followed by another glycine [11]. They are the most conserved positions and essential for structural folding and stabilization of the proteins.

The overall primary action of plant defensins is to form pores on the membrane of target pathogens and increase membrane permeability [52]. They can resist temperatures near the boiling point without losing function and their structures are well maintained. Their ultra-stability is generally due to the cysteine scaffold of their structures [11]. Conserved amino acid residue analysis of other Brassica members revealed that only eight cysteine residues are strictly conserved among members of the Brassica family. It was reported that the significance of both basic residues and their asymmetric distribution over the molecular surface causes the amphiphilic properties of defensins. Hoover, *et al.* [17] reported the importance of basic residues for the antimicrobial activity. According to Sagehashi, *et al.* [42], the site directed mutational analysis of Rs-AFP2, a defensin isolated from radish seeds showed that basic amino acid residues contribute to the antifungal potency of this peptide. The substitutions of neutral residues Gly or Val by arginine increased the Rs-AFP2 antifungal activity. However, according to Lay and Anderson [23], comparison of a series of sequences of plant defensins from various plant species reveals that the plant defensin family shows very limited sequence conservation except for eight cysteine, two glycine and one glutamate acid residues.

Gene Structure construction and prediction of *cis*-acting elements

To gain further insight into the structural evolution of the defensin genes in *B. juncea*, the exon-intron organization was analyzed by comparing the corresponding genomic sequences with their coding sequences through Gene Structure Display software. Based on the predicted schematic structures, all the genes displayed that a single intron and two exons existed in all the coding sequences (Figure 5). This conserved exon number among all plant defensins in *B. juncea* revealed their close evolutionary relationship. As described by Heyn, *et al.* [16], the overall rate of transcript production is determined by transcription and RNA processing rates. Gene architectural features, primarily the number and length of exons and introns have recently emerged as important regulatory players. Several new studies indicated that rapidly cycling cells contain gene-architecture toward short genes with few introns, allowing efficient expression during short cell cycles and in genes that need rapid regulation during stress. Therefore, the conserved trend toward shorter gene with fewer introns in plant defensins indicates their rapid induction following pathogen attack.

In plants, gene transcription is regulated by *cis*-acting regulatory elements that bind to target transcription factors. Some *cis*-regulatory elements are involved in stress responses. To analyze how the expression level of *B. juncea* defensins respond to stress stimuli, 2.0 kb upstream promoter regions of *B. juncea* defensin genes were scanned for stress-related *cis*-regulatory elements using the plant CARE online service. Two *cis*-acting regulatory elements involved in MeJA responsiveness, CGTA-motif and TGACG-motif were identified in the promoter region of *B. juncea* defensin genes denoting to the possible regulation of plant defensin genes under JA responsive pathway. Two light responsive elements GTI and a G-box, TGA-element in auxin responsiveness were identified indicating that these defensin genes might be involved in the response to various light stress and hormone treatments via participating in different regulatory mechanisms. Apart from that, 10 common *cis*-acting elements in promoter and enhancer region, and 14 core promoter elements around -30 of transcription start site were identified. The type and number of *cis*-acting regulatory elements in the promoter region of each *B. juncea* defensin gene were discrepant indicating that the members of *B. juncea* defensin gene family might be able to respond to different abiotic stresses. Similar pattern was observed in *cis*-acting regulatory elements in *C. sativa* defensin genes.

Phylogenetic analysis of defensin proteins of *B. juncea*, *C. sativa* and related species

A phylogenetic tree was constructed on the basis of full length *B. juncea* defensin protein sequences to understand the phylogenetic relationship among these proteins (Figure 6). According to the MEGA software, the evolutionary history was inferred using the Neighbor-Joining method [43]. The optimal tree with the sum of branch length = 3.45580578 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [57] and are in the units of the number of amino acid substitutions per site. The proportion of sites where at least one unambiguous base is present in at least one sequence for each descendent clade is shown next to each internal node in the tree. The analysis involved 6 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 77 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The tree indicates that *B. juncea* defensins belong to three distinct groups, majority of defensins clustered under group I. However, the defensin protein sequences of *C. sativa* used in phylogenetic analysis resulting in the categorization into five distinct groups (Figure 7).

Figure 6: The phylogenetic tree of *B. juncea* defensin proteins constructed by Neighbor-Joining method.

Figure 7: The phylogenetic tree of *C. sativa* defensin proteins constructed by Neighbor-Joining method.

To study the evolutionary relationship of *B. juncea* and *C. sativa* to other related species comprehensively, defensin proteins of *B. napus*, *B. oleracea*, and *B. rapa*, *B. nigra* were also obtained from BRAD database. A neighbor-joining tree was constructed with defensin proteins of *B. juncea* and other related species including *B. napus*, *B. oleracea*, *B. rapa* and *A. thaliana* (Figure 8). Phylogenetic analysis showed that all the identified defensins from Brassica spe-

Expression analysis of defensin genes in *B. juncea* and *C. sativa* in response to SA, JA and *A. brassicae* infection and wounding

To study the role of plant defensin in defense of *B. juncea* and *C. saiva*, induction of defensin genes in response to exogenous application of SA and JA was studied by performing qRT-PCR on RNA

ing plant defensins and thionins [40]. Expression analysis of defensin revealed that both the genotypes respond to JA application. In *B. juncea* the expression of defensin gene increase gradually up to 12 h and then with a sharp increase, peaked at 48 h after application. After 48 h, the expression of the gene was declined (Figure 9). However, in *C. sativa* the expression was early compared to *B. juncea*, revealing a sharp peak at 6 h after JA application, and then down-regulated at each time point after treatment. Presence of two *cis*-acting regulatory elements involved in MeJA responsiveness in promoter region of defensin genes further support the theory of regulation of defensins by JA signaling. Chamil., *et al.* [4] also reported that upon JA treatment, increased expression of *PR3*, *PDF1.2*, and *GST* was observed. This is in agreement with *Arabidopsis* defense signaling pathway that JA generally induces *PDF1.2* and *PR3* and therefore, those two genes have been extensively used as markers for JA signaling pathway [51].

Figure 9: Real-time PCR analysis of defensin genes in *C. sativa* and *B. juncea* in response to exogenous application of JA shows differential expression.

However, unlike JA, there was a significantly low level of defensin gene expression in response to exogenous SA application in both the genotypes (Figure 10). The decrease in defensin gene expression by SA coincides with the increased expression by JA confirming the antagonistic interaction between the SA and JA response pathways. Past literature revealed that JA responsive genes such as *PDF1.2* are highly sensitive to suppression by exogenous application of SA [20,40,45].

Many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent resistance over JA-dependent defense. This antagonism between SA and JA signaling was observed in a large number

of *Arabidopsis* accessions, highlighting the potential significance of SA/JA crosstalk in nature [6]. Though many reports describe SA/JA antagonism, synergistic interaction have been reported as well [34]. Emerging data suggests that, there is a more complex signaling network evoking both positive and negative regulatory interactions [28]. Furthermore, analysis of some *Arabidopsis* mutants with constitutive SA responses reveals a pathway in which JA and ET signaling are required for SA responses [54]. Mur., *et al.* in 2006 reported that in *Arabidopsis* treatments with low concentrations of JA and SA resulted in a synergistic effect on the JA and SA responsive genes *PDF1.2* and *PR1*, respectively. However, at higher concentrations the effects were antagonistic, demonstrating that the outcome of the SA-JA interaction is dependent on the relative concentration of each hormone. Chamil., *et al.* [4] also reported that although *PDF1.2* is known to be induced by JA but not by SA in *Arabidopsis*, *PDF1.2* could also be induced by SA in *C. sativa*. The JA-responsive genes *LOX2*, *VPS* and *PDF1.2* contain one or more SA responsive TGACG motifs in their promoters. The occurrence of this motif in defensin gene promoter indicates a cross talk between SA and JA pathways [2].

Figure 10: Real-time PCR analysis of defensin genes in *C. sativa* and *B. juncea* in response to exogenous application of SA a down-regulation.

In order to study the expression of defensin gene to *Alternaria* infection, plants were infected with a suspension of *A. brassicae* spores. Quantitative real time PCR analysis indicated that, relative expression of defensin gene in *C. sativa* in response to *Alternaria* infection is comparatively very low in spite of the genotypes' resistance to *A. brassicae* (Figure 11). The gene induction was very marginal. This can be explained by studying other defensin genes which are many [56] in case the gene in consideration in this study may not involve in resistance. Also might be important are levels of SA and JA in *C. sativa* which needs to be explained. However, Chamil., *et al.* [4] demonstrated a remarkable induction of *PDF1.2* in locally

challenged leaves of *C. sativa* in response to *A. brassicae* infection. However, there was a marked increase in defensin gene expression in *B. juncea* compared to *C. sativa* in response to infection. The relative expression was highest at 24 h, continue till 48 h, and then decreased. According to Pieterse., *et al.* [40], biotrophic pathogens are generally sensitive to defense responses that are regulated by SA, whereas pathogens with a necrotrophic lifestyle are commonly deterred by defenses that are controlled by JA and ET. An *Arabidopsis* defensin, activated after necrotrophic fungi invasion, is encoded by *PDF1.2* gene and is a predominant marker gene for JA pathway [51]. Since *A. brassicae* is a necrotrophic pathogen, it is obvious that induction of defensin by the pathogen as defensin gene is in JA signaling pathway.

Figure 11: Real-time PCR analysis of defensin genes in *B. juncea* and *C. sativa* in response to a *Alternaria* infection.

Two genotypes responded differentially to wounding. Expression of defensin gene in wounded *C. sativa* leaves increased initially, showing maximum fold change at 6h, and then decreased gradually while in *B. juncea* there was a marked decline of the expression from the initial point (Figure 12).

Figure 12: Real-time PCR analysis of defensin genes in *B. juncea* and *C. sativa* in response to wounding.

According to Dong [7] the wound induced octadecanoid pathway results in the synthesis of the signal molecule JA, and subsequent activation of genes in defense including defensin. Wounding not only causes a rapid production of JA but the level of ethylene as well [39]. Wounding and JA evoke a very similar response in *C. sativa* in our study further strengthening the idea that wounding exerts its effect through JA signaling. There are two distinct pathways for synthesis of JA. One starts from *PLD* (phospholipase D) induce by wound, pest, pathogen, elicitors, and the other starts from *DAD1* (defective anther dehiscence1) in developmental pathway lead to synthesis of AOC (allene oxide cyclase). Growing evidence indicates that developmentally regulated JA biosynthesis in *Arabidopsis* is controlled through activation of a JA biosynthetic pathway that differs from, but overlaps with the biosynthetic pathway that regulates wound-induced JA biosynthesis [54].

Conclusion

In-silico analysis and characterization of defensin genes in *B. juncea* and *C. sativa* leads to a better understanding of their roles in response to biotic and abiotic stresses. The wild Camelina poses a significantly higher number of defensin genes compared to Brassica and the proteins were more stable. This may be a possible reason of Camelina's resistance to *Alternaria* blight. Presence of *cis*-acting regulatory elements involved in MeJA responsiveness indicates their expression in response to stress conditions. Analysis of defensin gene response to SA, JA, *Alternaria* infection, and wounding revealed a complex and interesting network of pathways that function both separately and together to render defense responses in both the genotypes. The defensin genes can be used for further studies including antimicrobial studies to find effectiveness of using defensin in transgenic Brassica to confer resistance to *A. brassicae*.

Acknowledgments

Authors wish to acknowledge the support given by National Phytotron Facility for providing glasshouse facilities. Chaturani GDG acknowledge the receipt of PhD fellowship from Indian Council for Agricultural Research.

Bibliography

1. Anderson CR., *et al.* "Transcriptional control of plant defense gene expression". *Australian Biochemist* 31.4 (2000): 8-11.
2. Anuradha TS., *et al.* "A defensin gene of Indian mustard is stress induced". *Journal of Plant Biochemistry and Biotechnology* 8.2 (2009): 221-224.

3. Carvalho AO and Gomez VM. "Plant defensins – Prospects for the biological functions and biotechnological properties". *Peptides* 30 (2009): 1007-1020.
4. Chamil NNM, et al. "Defense gene induction in *C. sativa* upon *Alternaria brassicae* challenge". *Indian Phytopathology* 67.3 (2014): 252-256.
5. Colilla FJ, et al. "g-Purothionin: Amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm". *FEBS Lettres* 270: 191-194.
6. Does DV, et al. "Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59". *The Plant Cell* 25 (2013): 744-761.
7. Dong X. "SA, JA, ethylene, and disease resistance in plants". *Current Opinion in Plant Biology* 1 (1998): 316-323.
8. Donnes P and Hoglund. "A Predicting protein subcellular localization: Past present and future". *Genome Protein Bioinformatics* 2.4 (2004): 209-245.
9. Ebrahim S, et al. "Pathogenesis related (PR) proteins: chitinase and β -1,3-glucanase in defense mechanism against malformation in mango (*Mangifera indica* L.). *Scientia Horticulturae* 130.4 (2011): 847-852.
10. Eppl P, et al. "An Arabidopsis thaliana thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins". *Plant Physiology* 109 (1995): 813-820.
11. Gachomo EW, et al. "Structural characterization of plant defending protein superfamily". *Molecular Biology Reports* 39.4 (2012): 4461-4469.
12. Gasteiger E, et al. "The Proteomics Protocols Handbook, Humana Press". (2005): 571-607.
13. Ghose K, et al. "Differential profiling of selected defense related genes induced on challenge with *Alternaria brassicola* in resistant white mustard and their comparative expression pattern in susceptible Indian mustard". *Molecular Plant Pathology* 9.6 (2008): 763-775.
14. Glazebrook J. "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens". *annual review of phytopathology* 43 (2005): 205-227.
15. Hanfrey C, et al. "Leaf senescence in *Brassica napus*: Expression of genes encoding pathogenesis related proteins". *Plant Molecular Biology* 30.3 (1996): 597-609.
16. Heyn P, et al. "Introns and gene expression: Cellular constraints, transcriptional regulation, and evolutionary consequences". *Bioassays* 37 (2015): 148-154.
17. Hoover DM, et al. "Antimicrobial Characterization of Human β -Defensin 3 Derivatives". *Antimicrobial agents Chemother* 47 (2003): 2804-2809.
18. Kaewklom S, et al. "Structural and biological features of a novel plant defensin from *Brugmansia x Candida*". *Plos One* 13.8 (2018): e0201668.
19. Keller H, et al. "Pathogen induced elicitor production in transgenic tobacco generates a hypersensitive response and non-specific disease resistance". *Plant Cell* 11 (1999): 223-235.
20. Koornneef A, et al. "Kinetics of salicylate mediated suppression of jasmonate signaling reveal a role for redox modulation". *Plant Physiology* 147 (2008): 1358-1368.
21. Kumar D, et al. "Alternaria blight of oilseed Brassicas: A comprehensive review". *African Journal of Microbiology Research* 8.30 (2014): 2816-2829.
22. Lacerda AF, et al. "Antifungal defensins and their role in plant defense". *Front Microbiology* 5.116 (2014): 1-10.
23. Lay FT and Anderson MA. "Components of the innate immune system in plants". *Current Protein and Peptide Science* 6.1 (2005): 85-101.
24. Lay FT, et al. "Isolation and properties of floral defensin from ornamental tobacco and petunia". *Plant Physiology* 131 (2003): 1283-1293.
25. Leon IP and Montesano M. "Activation of defense mechanisms against pathogens in mosses and flowering plants". *International Journal of Molecular Sciences* 14.2 (2013): 3178-3200.
26. Liu J and Wang XJ. "An integrative analysis of the effects of auxin on jasmonic acid biosynthesis in *Arabidopsis thaliana*". *Journal of Integrative Plant Biology* 48 (2006): 99-103.
27. Liu YJ, et al. "Solution structure of the plant defensin VrD1 from mung bean and its possible role in insecticidal activity against bruchids". *Proteins: Structure, Function, and Bioinformatics* 63 (2006): 777-786.

28. Loake G and Grant M. "Salicylic acid in plant defense- the players and protagonists". *Current Opinion in Plant Biology* 10 (2007): 466-472.
29. Loon LCV and Strien EA. "The families of pathogenesis related protein, their activities, and comparative analysis of PR-1 type proteins". *Physiological and Molecular Plant Pathology* 55.2 (1999): 85-97.
30. Maarof E., *et al.* "Cloning and heterogeneous expression of CDef1, a ripening induced defensin in *Capsicum annum*". *Australian Journal of Crop Science* 5.3 (2011).
31. Mendez E., *et al.* "Primary structure and inhibition of protein synthesis in eukaryotic cell free system of a novel thionin, gamma-thionin, from barley endosperm". *European Journal of Biochemistry* 194 (1990): 533-539.
32. Mishra A., *et al.* "Molecular cloning and in-silico analysis of functional homologous of hypersensitive response genes induced during pathogenesis of Alternaria Blight in two genotypes of Brassica". *Journal of proteomics and bioinformatics* 3 (2010): 244-248.
33. Moosa A., *et al.* "Transgenic expression of antifungal pathogenesis-related proteins against phyto-pathogenic fungi- 15 years of success". *Israel Journal of Plant Sciences* 6 (2017): 122-134.
34. Mur LAJ., *et al.* "The outcomes of concentration specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death". *Plant Physiology* 140 (2006): 249-262.
35. Nayanakantha NMC., *et al.* "Differential expression of defense-related genes in *Synopsis alba* and *Brassica juncea* upon the infection of *Alternaria brassicae*". *Tropical Agricultural Research* 27.3 (2016): 123-136.
36. Newman T., *et al.* "Genes galore: A summary of the methods for accessing the results of large scale partial sequencing of anonymous *Arabidopsis thaliana* cDNA clones". *Plant Physiology* 106 (1994): 1241-1255.
37. Nowicki M., *et al.* "Alternaria black spot of Crucifers: Symptoms, importance of disease, and perspectives of resistance breeding". *Transgenic Research* 19 (2012): 373-384.
38. Oomen RJFJ., *et al.* "Plant defensin AhPDF1.1 is not secreted in leaves but it accumulates in intracellular compartments". *New Phytologist* 192.1 (2011): 140-150.
39. Pieterse CMJ and Loon LC. "Salicylic acid independent plant defense pathways". *Trends in Plant Science* 4.2 (1999): 52-58.
40. Pieterse CMJ., *et al.* "Networking by small molecule hormones in plant immunity". *Nature Chemical Biology* 5.5 (2009): 308-316.
41. Rawat S., *et al.* "Expression analysis of chitinase upon challenge inoculation to *Alternaria* wounding and defense inducers in *Brassica juncea*". *Biotechnology Reports* 13 (2017): 72-79.
42. Sagehashi Y., *et al.* "Purification and cDNA cloning of a defensin in *Brassica juncea*, its functional expression in *Escherichia coli*, and assessment of its antifungal activity". *Pesticide Science* 38.1 (2013): 33-38.
43. Saitou N and Nei M. "The neighbor-joining method: a new method for reconstructing phylogenetic trees". *Molecular Biology and Evolution* 4.4 (1987): 406-425.
44. Saskia CM., *et al.* "Characterization of the early response of *Arabidopsis* to *Alternaria brassicae* infection using expression profiling". *Plant Physiology* 132 (2003): 606-617.
45. Spoel SH. "NPR1 modulates cross-talk between salicylate and jasmonate dependant defense pathways through a novel function in the cytosol". *Plant Cell* 15 (2003): 760-770.
46. Sticher L., *et al.* "Vacuolar chitinases of tobacco: a new class of hydroxyproline containing proteins". *Science* 257 (1992): 655-657.
47. Stotz HU., *et al.* "Plant defensins: defense, development and application". *Plant Signal Behave* 4 (2009): 1010-1012.
48. Tam JP., *et al.* "Antimicrobial peptides from plants". *Pharmaceuticals* 8.4 (2015): 711-757.
49. Thevissen K., *et al.* "A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (*Dahlia merckii*)". *Proceedings of the National Academy of Sciences of the United States of America* 07 (2000): 9531-9536.
50. Thevissen K., *et al.* "Interaction of antifungal plant defensins with fungal membrane components". *Peptides* 24 (2003): 1705-1712.
51. Thomma BPHJ., *et al.* "Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate". *Plant Physiology and Biochemistry* 38.5 (2000): 421-427.

52. Thomma B., *et al.* "Mode of action of plant defensins suggests therapeutic potential". *Current Drug Targets - Infectious Disorders* 3.1 (2003): 1-8.
53. Tiwari S., *et al.* "Effect of methyl jasmonate on disease severity and expression of plant defensin gene during *Alternaria brassicae* infection in *Arabidopsis*". *International Journal of Current Microbiology and Applied Sciences* 6.7 (2017): 857-865.
54. Turner JG., *et al.* "The jasmonate signal pathway". *The Plant Cell* (2002): 153-164.
55. Zhao KJ and Chye ML. "Methyl jasmonate induces expression of a novel *Brassica juncea* chitinase with two chitin binding domains". *Plant Molecular Biology* 40.6 (1999): 1009-1018.
56. Zhou P. "Determining protein half-lives. (In): Dickson, R. C. and Mendenhall, M. D. (eds). Signal transduction protocols". *Methods in Molecular Biology* 284 (2004).
57. Zuckerkandl E and Pauling L. "Evolutionary divergence and convergence in proteins". *Evolving Genes and Proteins* (1965): 97-166.

Volume 3 Issue 12 December 2019

© All rights are reserved by Anita Grover., *et al.*