VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 376-383

Isolation and Characterization of Defensins Genes from Vietnamese *Brassica juncea*

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Received 15 July 2016
Revised 25 August 2016; Accepted 09 September 2016

Abstract: Plant defensins are small, basic cysteine-rich peptides ranging from 45 to 54 amino acids and are positively charged. They are a part of the innate immune system and possess antifungal and/or antibacterial activities found in many plant species, including Brassica family. *Brassica juncea* has been known as a nutritional vegetable, medicinal plant species and an oilseed crop in many countries. It has also been reported to be heat- and drought-tolerant and resistant to fungal diseases. This study aimed to isolate and characterize the *BjAFP* defensins genes from Vietnamese *B. juncea*. As the result, three nucleotide sequences of defensins genes were amplified, including *BjAFP1*, *BjAFP4*-like and an unpublished gene called *BjAFPx*. By comparison of DNA sequences from PCR and RT-PCR products, the result showed that, each gene consisted of one intron and two exons. Two exons had respectively 64 nucleotides and 179 nucleotides while intron contains 91 nucleotides in *BjAFP1*, 93 nucleotides in *BjAFP4* and 98 nucleotides in *BjAFPx*. Besides, gene *BjAFP1* expressed at transcription level in all tissues: stem, root, leaf, flower and seed of *B. juncea*. In comparison of the nucleotide sequence of *BjAFP1* with two published sequences of this gene in Genbank, the single nucleotide polymorphisms in *BjAFP1* have been identified, including one missense substitution at position 54 in nucleotide sequence, which replcaed amino acid phenylalanine by leucine, and three synonymous at positions 51, 204 and 225 in nucleotide sequence.

Keywords: Brassica juncea, defensin, BjAFP1, BjAFP4, BjAFPx.

1. Introduction

Small antimicrobial peptides play an important role as part of the plants' natural defense system against infectious microorganisms, by recognizing a broad range of microbes. Hundreds of antifungal peptides and proteins are known, with more being discovered almost daily. Defensins are one of the main groups of antimicrobial peptides found in plants. They also have functions as α-amylase inhibitors, protease inhibitors, protein synthesis inhibitors as well as roles in heavy metal tolerance and development [1]. Plant defensins are widely distributed in a vast majority of plant families, including Brassicaceae [2-3]. The plant defensins family is quite diverse regarding amino acid composition as only the eight structure stabilizing cysteines appear to be conserved among all plant defensins [4]. The variation in

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the primary sequences may account for the different biological activities reported for plant defensins. The three dimensional structure of plant defensins comprises a triple-stranded β-sheet with an α-helix in parallel, stabilized by four disulfide bridges [5]. Not all plant defensins have the same mode of action. Some of them exhibit potent antifungal activity *in vitro* at micromolar concentrations against a broad spectrum of filamentous fungi.

The isolation and characterization of a wide range of defensins peptides are crucial for the continued development of economically and medically important products. Analysis of the sequenced plant genomes revealed that defensins are present as multigene families and are overrepresented in the genomes of some plants species [6]. Nowadays, the number of defensins sequences is much more than 350, which has been deposited in the protein database at the National Centre for Bioinformatics (NCBI). The plant defensins Rs-AFP1 and Rs-AFP2 from radish (*Raphanus sativus*), and alfAFP isolated from seeds of the *Medicago sativa* (alfalfa) plants [7], are examples of potent antifungal proteins, causing morphological distortions of the fungal hyphae, resulting in hyperbranched fungal structures.

There are some reports on defensins genes of *Brassica juncea* which have been published. *BjAFP1* gene sequences have been identified and submitted in Genbank. Swathi T. et al. (India) have reported that a full-length defensin gene *BjAFP1* has 956 bp in length, with an open reading frame of 243 bp capable of coding for a peptide of 80 amino acids [8] (Genbank: DQ191752.1). Hiroaki Takaku et al. (Japan) have submitted a DNA fragment of 156 bp of *BjAFP1* coding sequence on Genbank (Genbank: AB537492.1). Comparison of two above sequences showed two polymorphisms, which are 204T>C and 225C>T in the coding sequence of *BjAFP1* (numbering according to nucleotide sequence submitted by Swathi et al.). Therefore, they are probably two single nucleotide polymorphisms of *BjAFP1*.

With the wealth of defensins nucleotide sequences available, strategies of gene isolation coupled with recombinant production are increasingly been used for the characterization of closely related plant defensins peptides. Therefore, this study presents the identification of defensins genes from Vietnamese *Brassica juncea*, based on the sequence homology exists within the nucleotides encoding defensins from domesticated Brassicaceae species published in Genbank.

2. Materials and methods

2.1. Plant material

Seed sample of *Brassica juncea* (seed) obtained from Vietnam Plant Resources Center was germinated and grown to collect the root, stem, leaf and flower samples. These tissues were collected and stored at -20^{o}C for further investigation of defensins gene isolation.

2.2. Extraction of total DNA and RNA from B. juncea

Total DNA was extracted by using CTAB method modified from Hombergen and Bachmann in 1995 [9], stored at -20˚C for further investigation. Total RNA from five tissues of *B. juncea* plant (leaf, flower, stem, seed, root) was extracted by using Thermo Scientific GeneJET Plant RNA Purification Mini Kit, then treated with DNase I to remove genomic DNA.

2.3. Amplification of defensins genes from total DNA template (PCR)

In this study, DNA fragments containing defensins genes (*BjAFP*s) were amplified by PCR, using 3 pairs of primers (Table 1). These primers were designed based on the nucleotide sequences of promoter and complete coding region submitted in Genbank of *BjAFP1* gene by Swathi T. et al. (Genbank: EU418763.1 and DQ191752.1) and *BjAFP4* gene by Rawat S. et al. (Genbank: KF578144.1).

Table 1. Primers using for PCR to amplify defensin genes in *B. juncea*

No.	Sequence of primers	Annealing temperature	Product length
1	Fw1: 5'CAGTCGTTTAGCCACCGAGT 3' Rv1: 5'GAAGTAGCAGATACACTTGTGAGC 3'	52 ˚C	~600 bp (*BjAFP1*)
2	Fw4: 5'GTGGTGGAGAAACCAGCCA 3' Rv4: 5'GCACTACAGAGTTTTGTTAGACCA 3'	56 ˚C	~600 bp (*BjAFP4*)
3	Fw4: 5'GTGGTGGAGAAACCAGCCA 3' Rv1: 5'GAAGTAGCAGATACACTTGTGAGC 3'	54 ˚C	*BjAFPx*

2.4. RT-PCR for amplification of coding region of BjAFP1 gene

cDNA was synthesized in the first step of RT-PCR (reverse transcription) by using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. The product of the first strand cDNA synthesis was directly used in PCR to amplify *BjAFP1* gene using primers designed based on the coding sequence of *BjAFP1* (Genbank: DQ191752.1) that was submitted by Swathi T. et al. in Genbank by using Primer BLAST tool on NCBI website.

Table 2. Primers using for RT-PCR to amplify *BjAFP1* from total RNA in *B. juncea*

Sequence of primers	Annealing temperature	Product length
Fw3: 5'GTTGCTTCCATCATTGCCCTAC 3' Rv1: 5'GAAGTAGCAGATACACTTGTGAGC 3'	56˚C	~230 bp

2.5. DNA sequencing and data analysis

PCR and RT-PCR products were purified by using Thermo Scientific GeneJET Gel extraction Kit and Bioneer AccuPrep PCR Purification Kit respectively. Purified PCR product*s* were sent to FirstBASE Laboratory for DNA sequencing. Nucleotide and amino acid sequences were analyzed and aligned by software on NCBI website and ClustalW2 software. The amino acid sequence was predicted by using Translator software on http://www.fr33.net/translator.php.

3. Results and discussion

3.1. DNA and RNA extraction

The extracted total DNA was analyzed by electrophoresis on 1% agarose gel in TAE 1X buffer as shown in Figure 1.

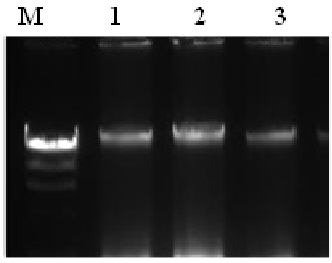

Figure 1. Result of electrophoresis on 1% agarose in TAE 1X buffer of total DNA extracted from *B. juncea* leaves. Lane M: Marker 1 kb, Lane 1, 2, 3: repeated leaf samples.

Total RNA was extracted by using Thermo Scientific GeneJET Plant RNA Purification Mini Kit. After digestion with DNase I, RNA product was checked by electrophoresis on 1% agarose gel in TAE 1X buffer. As shown in the figure 2, the ratio of 28S rRNA:18S rRNA was almost 2:1 in all samples; this indicated that the RNA was completely intact.

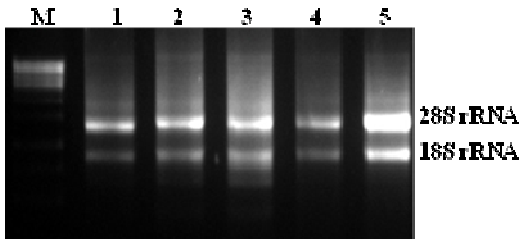

Figure 2. Result of electrophoresis on 1% agarose gel in TAE 1X buffer of RNA extracted from 5 tissues of *Brassica juncea*. M: marker 1kb. 1, 2, 3, 4, 5: RNA extracted from 5 tissues: seed, stem, leaf, root and flower respectively.

3.2. PCR and RT-PCR for amplification of B. juncea defensins genes

As the results shown in Figure 3 (A and B) and Figure 4, amplicons appeared as only unique bright bands in each PCR or RT-PCR. Figure 3A and Figure 4 respectively showed that the genomic DNA sequence length of *BjAFP1* was approximately 600 bp and the length of cDNA fragment of *BjAFP1* was over 200 bp while the sequence length of genomic DNA of *BjAFP4* and *BjAFPx* were nearly 600 bp as expected. In addition, RT-PCR result as shown in the Figure 4 indicated that *BjAFP1* gene expressed in all five organs of mustard plant: leaf, flower, seed, root and stem.

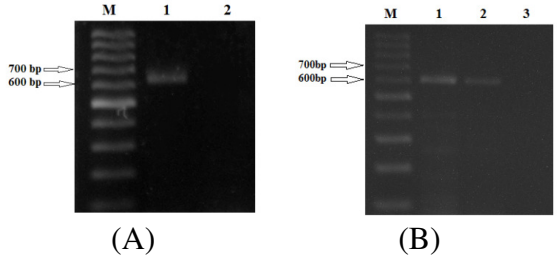

Figure 3. Result of electrophoresis on 2% agarose gel in TAE 1X buffer of PCR products from genomic DNA for amplification of (A) *BjAFP1* (M: Marker 100bp, 1: PCR product of *BjAFP1*, 2: Negative control) and (B) *BjAFPx* (Lane 1), *BjAFP4* (Lane 2) genes, M: Marker 100bp and 3: Negative control.

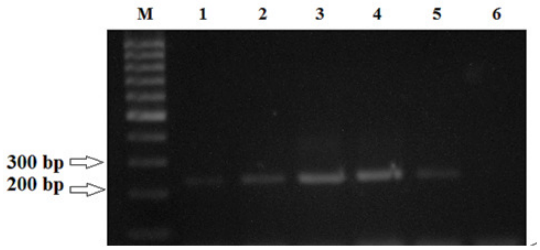

Figure 4. Result of electrophoresis on 2% agarose gel in TAE 1X buffer of cDNA fragments of *BjAFP1* amplified by RT-PCR. M: Marker 100 bp. 1, 2, 3, 4 ,5 are RT-PCR products from stem, flower, leaf, seed, root samples respectively. 6: Negative control.

3.3. DNA sequencing and analysis

All PCR and RT-PCR products were sequenced and the results were compared with published data on Genbank to identify the homology as well as the position of exons and introns by comparing the genomic DNA sequence and cDNA sequence.

Table 3. The predicted single nucleotide polymorphisms in *BjAFP1* gene compared to

No.	SNP alleles	SNP position in coding sequence	Amino acid change
1	T/G	51	No
2	T/A	54	Leucine/Phenylalanine
3	T/C	204	No
4	T/C	225	No

The nucleotide sequence of cDNA from leaf sample was compared with two coding sequences of *BjAFP1*, code DQ191751.1 (Swathi T. et. al, India) and AB537492.1 (Takahu H. et al, Japan) submitted in Genbank. There are 4 single nucleotide polymorphisms between cDNA sample in this study and two sequences submitted in Genbank and these SNP site were listed in Table 3. The differences at position 51, 204 and 225 were both synonymous changes and did not lead to affect

the amino acid sequences of *BjAFP1* defensin protein. However, the difference at position 54 led to substitute amino acid – phenylalanine (F) by leucine (L) in BjAFP1 defensin peptide (Figure 5).

DNA sequencing result of RT-PCR products of BjAFP1 cDNA from all tissue samples showed that, there was one position containing two peaks at a position of nucleotides, indicating that the genotypes of *BjAFP1* in those individual samples were heterozygous.

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CLUSTAL 2.1 multiple sequence alignment

GeneBank_ABB59548.1_Swathi_anu      MAKVASIIALLFAALVLFAAFEAPTMVEAQKLCERPSGTWSGVCGNNNAC  50
GeneBank_BAJ41194.1_Takaku_H.       ----------------------------QKLCERPSGTWSGVCGNNNAC
cDNA_leaf                           ---VASIIALLFAALVLLAAFEAPTMVEAQKLCERPSGTWSGVCGNNNAC
                                                                ********************

GeneBank_ABB59548.1_Swathi_anu      KNQCINLEKARHGSCNYVFPAHKCICYFPC                      80
GeneBank_BAJ41194.1_Takaku_H.       KNQCINLEKARHGSCNYVFPAHKCICYFPC
cDNA_leaf                           KNQCINLEKARHGSCNYVFPAHKCICYF--
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Figure 5. The comparison of predicted amino acid sequence translated from leaf mRNA of *BjAFP1* and *BjAFP1* amino acid sequences submitted in Genbank (ABB59548.1 by Swathi T. et al.and BAJ41194.1 by Takaku H. et al.). Alignment was created in ClustalW2.

In addition, alignment of nucleotide sequence of amplified *BjAFP4* with *BjAFP4* sequence from Genbank (ID code: KF578144.1 by Rawat S. et al., India) by BLAST showed that *BjAFP4* sequence from Indian *B. juncea* was 100% identical with the sequence of *BjAFP4* from *B. juncea* in Vietnam. Because of complete homology, it could be deduced that the nucleotide sequence of *BjAFP4* in our study contained two exons, which were respectively 64 and 179 nucleotides, and one intron of 93 nucleotides (Figure 6).

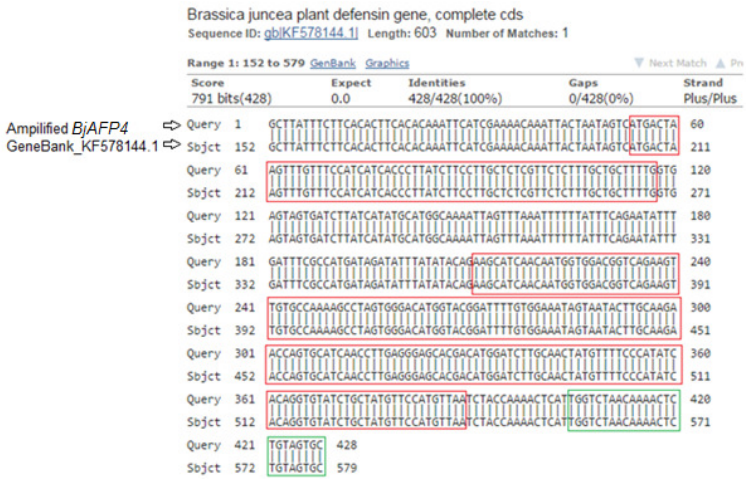

Figure 6. The comparison of nucleotide sequence of amplified *BjAFP4* from leaf samples and *BjAFP4* submitted in Genbank (KF578144.1 by Rawat S. et al., India). The reverse primer is marked by the frame and the open reading frame is marked by the other frames. Alignment was created by Align Sequences Nucleotide BLAST software on NCBI website.

In this study, another *BjAFP* gene also was successfully amplified by using the third pair of primer (Table 1) in PCR. By DNA sequencing and alignment of the nucleotide sequence of *BjAFPx* to *BjAFP1* and *BjAFP4*, these genes showed the high similarity and the coding sequence of *BjAFPx* can be predicted based on the known coding sequences of *BjAFP1* and *BjAFP4* (Figure 7). *BjAFPx* probably had 2 exons and 1 intron with the length of first exon, second exon and intron are 64 bp, 179 bp and 98 bp respectively.

Figure 7. The comparison of DNA nucleotide sequence of *BjAFPx* and *BjAFP4* revealed in this study and *BjAFP1* submitted in Genbank (DQ191752.1 by Swathi T. et al.). Alignment was created in ClustalW. The coding sequence of *BjAFP4*, *BjAFP1* and the predicted coding sequence of *BjAFPx* are respectively marked by the frames in the corresponding line.

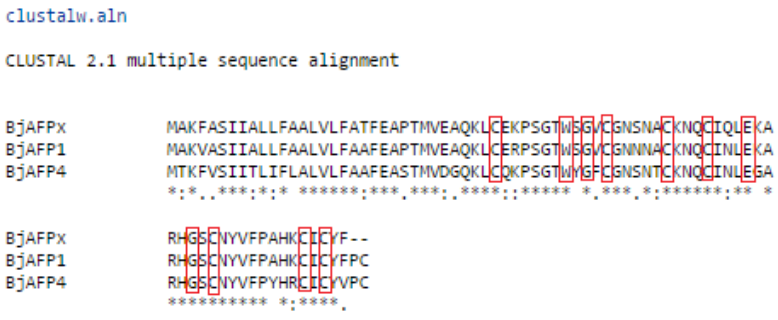

Figure 8. The comparison of predicted amino acid sequence coding by *BjAFPx* with amino acid sequence coding by *BjAFP4* and *BjAFP1* submitted in Genbank (ABB59548.1 by Swathi T. et al.). Alignment was created in ClustalW2. The conserve amino acids of defensin family are marked in red frame.

From the predicted coding sequence of *BjAFPx*, the amino acid sequence of BjAFPx peptide can be also predicted by using Translator software. Also, that predicted sequence of BjAFPx peptide was compared with the peptide sequence encoded by *BjAFP1*

and *BjAFP4* using ClustalW. The result shown in Figure 8 indicated the high similarity of amino acid sequence of BjAFPx, BjAFP1 and BjAFP4 peptides. In fact, BjAFPx amino acid sequence was 94% identical with BjAFP1 amino acid sequence when compared to both sequences by using Align Sequences Protein BLAST on NCBI website. In addition, BjAFPx peptide has conserved amino acids of defensins family so BjAFPx definitely belongs to plant defensins family.

4. Conclusion

In this study, 334 bp long *BjAFP1* gene amplified by PCR and 243 bp of cDNA from five different tissues, including root, stem, leaf, flower and seed of *Brassica juncea* had been amplified by RT-PCR techniques. Two other defensins genes, *BjAFP4* and an unpublished *B. juncea* defensins gene named *BjAFPx*, were also successfully identified.

Analysis of DNA sequences from genomic and cDNA sequence of these genes showed that each of *BjAFP1 or BjAFP4 or BjAFPx* defensins gene consisted of one intron and two exons. Two exons had respectively 64 nucleotides and 179 nucleotides while intron contained 91 nucleotides in *BjAFP1*, 93 nucleotides in *BjAFP4* and 98 nucleotides in *BjAFPx*.

Comparison of identified sequences revealed that there were four substitutions in *BjAFP1* gene, including one missense at position 54 in nucleotide sequence, which replaced amino acid phenylalanine by leucine, and three synonymous at positions 51, 204 and 225 in nucleotide sequence compared to published sequences on Genbank. In addition, predicted BjAFPx amino acid sequence is 94% identical with known BjAFP1 amino acid sequence which indicated that this could be a member of defensins family.

Acknowledgements

The authors would like to express sincere thanks Faculty of Biology, VNU University of Science for support and providing equipment and condition to this study.

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Phân lập và mô tả các gen defensin từ cải bẹ xanh *Brassica juncea*

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Tóm tắt: Họ defensin thực vật gồm các peptit nhỏ giàu axit amin cysteine, có kích thước từ 45 – 54 axit amin. Đây là thành phần của hệ miễn dịch tự nhiên ở thực vật và có hoạt tính kháng khuẩn, kháng nấm, được tìm thấy ở nhiều loài thực vật, trong đó có họ Cải (Brassica). Cải bẹ xanh *Brassica juncea* được biết đến là loài thực vật được sử dụng làm rau xanh, đồng thời cũng là loài dược thảo có nhiều công dụng. Nó cũng được biết đến là loài có tính chống chịu và có khả năng kháng nấm. Nghiên cứu này nhằm phân lập và mô tả các gen mã hóa defensin *BjAFP* từ loài cải này thu tại Việt Nam, sử dụng kỹ thuật PCR, RT-PCR và giải trình tự ADN. Kết quả cho thấy, có ba trình tự nucleotit của các gen defensin đã được xác định, bao gồm *BjAFP1*, *BjAFP4* đã được so sánh với các trình tự đã công bố trên Genbank, và một trình tự gen được tạm gọi là *BjAFPx*. So sánh trình tự ADN cho thấy, cả ba trình tự gen này, mỗi trình tự gen đều chứa một intron và hai exon. Hai exon có trình tự tương ứng dài 64 và 179 bp, trong khi intron là khác nhau với độ dài 91 bp, 93 bp và 98 bp ở các gen tương ứng *BjAFP1*, *BjAFP4* và *BjAFPx.* Với RT-PCR, nghiên cứu cũng xác định được gen *BjAFP1* được phiên mã ở tất cả các mô nghiên cứu, gồm thân, rễ, lá, hoa và hạt. So sánh trình tự nucleotit gen *BjAFP1* với trình tự đã công bố trên Genbank cho thấy có 4 vị trí thay thế nucleotit, trong đó có một thay thế nhầm nghĩa (thay thế phenylalanine bởi leucine) và ba thay thế đồng nghĩa ở các vị trí 51, 204 và 225 trong trình tự gen này.

Keywords: Brassica juncea, defensin, BjAFP1, BjAFP4, BjAFPx.