Avrupa Bilim ve Teknoloji Dergisi
Sayı 33, S. 79-87, Ocak 2022

Araştırma Makalesi

www.ejosat.com ISSN:2148-2683

European Journal of Science and Technology
No. 33, pp. 79-87, January 2022

Research Article

In silico Analysis of Ribosome-Inactivating Protein (Tritin) from Common Wheat Plants (*Triticum aestivum* L.)

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(First received 10 November 2021 and in final form 3 January 2022)

(**DOI:** 10.31590/ejosat.1021686)

ATIF/REFERENCE: Demirel, S., Usta, M. & Güller, A. (2022). *In silico* Analysis of Ribosome-Inactivating Protein (Tritin) from Common Wheat Plants (*Triticum aestivum* L.). *European Journal of Science and Technology,* (33), 79-87.

Abstract

Ribosome-inactivating proteins (RIPs) are one of the enzymes that inhibit protein synthesis after depurination of a specific adenine in ribosomal RNA. The tritin is one of type I RIPs that include RNA-N glycosidase domain from RIP family. In the present study, cDNA encoding tritin from leaves of wheat Kutluk-94 cultivar was isolated and cloned into pGEM-T Easy vector. The recombinant plasmid was sequenced. The different bioinformatics tools were used for assessment of tritin protein characteristics. A total of 38 tritin-like sequences were identified in some monocot plants. Results showed that tritin protein have conserved domain (Ricin-A) found in other RIPs associated with RNA N-glycosidase activity and shows chancing homology to the RIPs in other plant species. According to multiple sequence alignment, tritin has conserved amino acids which are crucial role in RNA N-glycosidase activity. Our study illustrates that results obtained from *in silico* analyses could provide a perspective to another researcher about molecular and structural properties of tritin protein.

Keywords: Ribosome-inactivating protein, Tritin, cDNA

Ekmeklik Buğday Bitkisinden Ribozom İnaktivite Eden Proteinin (Tritin) *in Silico* Analizi

Öz

Ribozom inaktive eden proteinler (RIP'ler) ribozomal RNA'da spesifik bir adeninin depürünasyonundan sonra protein sentezini baskılyan enzimlerdir. Tritin RIP ailesinden RNA-N glikosidaz domainine sahip tip I RIP'lerden biridir. Mevcut çalışmada Kutluk-94 buğday çeşidinin yapraklarından tritini kodlayan cDNA izole edildi ve pGEM-T Easy vektöre klonlandı. Recombinant plazmid sekanslandı. Farklı biyoinformatik araçlar tritin proteininin özelliklerinin değerlendirilmesi için kullanıldı. Bazı monokotil bitkilerde toplamda 38 tritin benzeri sekans tespit edildi. Sonuçlar tritin proteininin diğer RIP'lerde bulunan RNA N-glikozidaz aktivitesi ile ilişkili korunmuş domaine (Ricin-A) sahip olduğunu ortaya koydu. Çoklu sekans hizalamaya analizi tritinin RNA N-glikozidaz aktivitesinde hayati rol oynayan korunmuş amino asitlere sahip olduğunu göstermiştir. Bizim çalışmamızda *in silico* analizlerden elde edilen sonuçlar tritin proteinin moleküler ve yapısal özellikleri hakkında diğer araştırmacılara bilgi sağlayacaktır.

Anahtar Kelimeler: Ribozom inaktive eden protein, Tritin, cDNA

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1. Introduction

Fungi, bacteria and viruses are disease agents that affect the biochemical, physical, and genetic mechanisms of plants and cause changes in properties such as biomass, yield, and quality in plants worldwide. Valuable compounds synthesized by plants with their specific metabolic pathways are used in the prevention and treatment of disease caused by microorganisms (Calixto, 2000). Among these, ribosome-inactivating proteins or antiviral proteins obtained from plants have worked intensively because they provide resistance to diseases (Huang et al., 2008). RIPs are enzymes that irreversibly inhibit protein translation by depuration of rRNA. Though RIP genes are found in bacteria, fungi, and, even some insects, these genes seem to be more common in plants (Peumans et al., 2010, Peumans et al., 2014). RIPs are also found in 17 different plant families (Girbes et al., 2004, Stripe, 2004). Most of the RIPs are more common in families such as *Cucurbitaceae, Poaceae, Caryophyllaceae*, *Euphorbiaceae*, *Sambucaceae* and *Phytolaccaceae* (Girbes et al., 2004; Domashevskiy and Goss, 2015; Shang et al., 2016). Some researchers have revealed that RIPs are also widely found in fungal species such as *Hypsizigus marmoreus, Lyophyllum shimeji* and *Volvariella volvacea* (Yao et al., 1998; Lam and Ng, 2001a; Lam and Ng, 2001b). Liu et al., (2002) revealed the presence of RIP proteins in the algae *Laminaria japonica* A, while Lapadula and Ayub (2017) and Lapadula et al., (2013) revealed the available of different RIP genes in the genome of two mosquitoes.

The RIPs discovered so far are classified based on their physical properties which are named Type I, II, and III (Virgilio et al., 2010). Type I RIPs are proteins with a single polypeptide domain of approximately 30 kDa molecular weight with N-glucosidase activity (Stirpe, 2004) (Figure 1). The firstly type I RIP identified was obtained from American pokeweed and then, named as pokeweed antiviral protein (PAP) (Dallal and Irvin, 1987). A huge number of type I RIPs were isolated from plant families such as *Cucurbitaceae*, *Euphorbiaceae,* and *Fabaceae*. Type II RIPs, fairly toxic heterodimeric proteins are composed of two polypeptide subunits (A and B chains) (Figure 1). The A-chain is associated with RNA N-glycosidase activity, while the B-chain is a lectin-like peptide that transports it from the plasma membrane to the entrance of the A chain (Stripe, 2004; Olsnes and Pihl, 1973a, b). Type-2 RIPs are classified into two groups as toxic and non-toxic. Although several Type-2 RIPs like abrin, ricin, modeccin, viscumin and volkensin exhibit highly toxic properties, Type-2 RIPs like nigrin, iris lectin, cinnamomin and ebulin are not toxic (Zhu et al., 2018). Type-3 RIPs are inactive precursor polypeptides (Mundy et al., 1994). Type-3 RIPs with a molecular weight of approximately 60 kDa are less common than other Type-1 and Type-2 RIPs (Peumans et al., 2001). Type III RIPs have an N-terminal domain associated with the A domain of the RIPs and a C-terminal domain of the unknown function (Nielsen and Boston, 2001; Hey et al., 1995) (Figure 1). When the C terminal domain from Type III RIPs is removed, they exhibit similar characteristics to Type 1 RIPs in terms of enzymatic activity and charge (Krawetz and Boston, 2000).

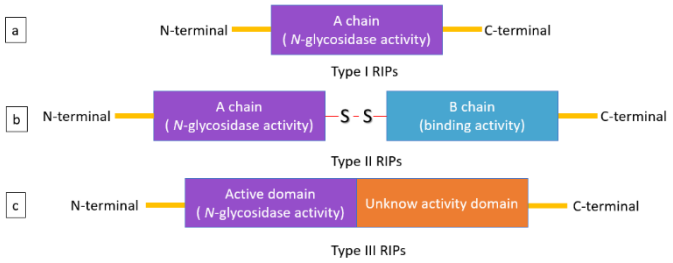

Figure 1. Diagrammatic representation of the structure of different of RIP types (Modified from Zhu et al., 2018)

RIPs have quite different enzymatic properties like N-glycosidase activity, DNase activity, lipase activity, chitinase activity (Endo et al., 1987; Shih et al., 1997; Lombard et a., 2001; Ruggiero et al., 2007). Due to these properties, RIPs exhibit various biological functions such as, antiviral, antibacterial and antifungal (Stirpe ve Battelli, 2006; Shu et al., 2009). Vivanco et al. (1999) reported that ME1 and ME2 RIPs obtained from *Mirabilis expansa* roots showed antibacterial activity against *Agrobacterium radiobacter, Agrobacterium tumefaciens* and *Pseudomonas syringea*. Researchers showed that several RIPs purified or isolated from plants such as *Nicotina tabaccum*, *Cucurbita moschata*, *Momardica balsamina*, *Mirabilis jalapa* have antibacterial activity (Sharma et al., 2004, Barbieri et al., 2006; Ajji et al., 2016; Rumiyati et al., 2014). RIPs have the potential to be used as plant defense agents against several fungal pathogens. TRIP (tabbacco RIP) was exhibited antifungal activity against different fungi pathogens involving *Fusarium oxysporum*, *Cochliobolus heterostrophus*, *Cytospora canker* and *Trichoderma reesei* (Sharma et al., 2004). MbRIP-1, diocin 2, luffacylin, alpha-momorcharin, and curcin 2 displayed antifungal activity against to several fungal pathogens by inhibiting their growth (Parkash et al., 2002, Zhu et al., 2013, Wang et al., 2012, Iglesias et al., 2016, Huang et al., 2007). A large number of studies have been indicated that RIPs have insecticidal activity against several insects including Coleptera, Diptera, and Lepidoptera (Wei et al., 2004, Kumar et al., 1993, Shahidi-Noghabi et al, 2008). Bertholdo-Vargas et al. (2009) signified that various type I RIPs reduce fecundity and survival when added to the diets of *Spodoptera frugiperda* and *Anticarsia gemmatalis* Hübner. Various RIPs of *Malus domestica* Borkh showed a highly aphicidal effect by reducing nymphal survival of *Myzus nicotianae* Blackman (Hamshou et al., 2016). There is a lot of literature on the antiviral properties of ribosome-inactivating proteins against plant and animal viruses. PAP was the first RIP shown to reduce *Tobacco mosaic virus* (TMV) infection by suppressing protein synthesis (Duggar and Armstrong, 1925; Dallal and Irvin, 1978). The external application of PAP increased systemic resistance to TMV infection in *N. benthamiana* (Zhu et al., 2016). Moroever, Sipahioğlu et al. (2017) demonstrated that PAP-I reduced the infection of *Zucchini yellow mosaic virus* in zucchini plants depending on its concentration. Choudhary et al. (2008) stated that BBAP1 obtained from *Bougainvilea xbuttiana* provided high resistance against TMV with N-glycosidase activity. Güller et al., (2018) reported that recombinant bouganin antiviral protein (BAP) from *Bougainvillea spectabilis* Willd reduced the severity of disease caused by ZYMV. Chen et al. (1991) found that 4 micrograms of PAP completely inhibited TMV infection in tobacco plants. Praveen et al., (2001) showed that single resistance inducing protein (Crip-31) from *Clerodendrum inerme* protected tobacco plants against RNA viruses such as *Cucumber*

mosaic virus (CMV), *Potato virus Y* (PVY), and TMV and inhibited more than 80% of the virus. The antiviral protein 2 (PIP2) of *P. insularis* plant has been displayed antiviral activity against TMV (Song et al., 2000). Zhu et al. (2013) revealed that α-MMC exhibits broad-spectrum antiviral activity against phytopathogenic viruses, including CMV, *Turnip mosaic virus* (TuMV), *Chilli veinal mottle virus* (ChiVMV) and TMV, and that α-MMC can activate systemic resistance against multiple virus infections.

There is no study about the tritin gene showing RIP function in Turkey. Therefore, in this study, we isolated tritin gene specific mRNA from *T. aestivum* cultivar Kutluk-94 and performed its molecular characterization and bioinformatics analysis.

2. Material and Metods

2.1. Plant Material and RNA Extraction

Kutluk-94 wheat cultivar seeds obtained from Eskişehir Transitional Zone Agricultural Research Institute were grown in the climate room of Department of Plant Protection of Van Yuzuncu Yil University. Fresh leaves were thoroughly ground and total RNA extraction from leaves was carried out according to the method reported by Foissac et al., (2001).

2.2. Amplification of Tritin Gene and Molecular Cloning

The cDNA synthesis was performed according to manufacture of a commercial kit (RevertAid First Strand cDNA kit, Vilnius, Thermo-Fermantas) using total RNA. The gene specific primers were designed based on the RIP gene sequences in the GenBank, NCBI (D13795.1) using SnapGene 5.1.7 software. Tritin-*EcoRI* F-5' CAGT*GAATTC*GATGGCGAAGAACGTGGACAA-3' and Tritin-*PstI* R-5' CAGT*CTGCAG*CTATTTCCCCCCCACTCTTATGA-3' primers were used for amplification of complete tritin gene. A total volume of 25 µl of PCR mixture contained; 0.5 µl of each primer (100 pmol), 2.5 µl of 10X reaction buffer, 0.5 µl of dNTPs (10 mM each), 1.5 µl of $MgCl_2$ (25 mM), 1.3 µl of cDNA, 0.2 µl of Thermo Taq DNA polymerase, and 18 µl of Nuclease free water. PCR reaction was carried out with the following cycling parameters: one cycle of pre-denaturation at for 95 for 2 min, 37 cycles of denaturation at 95 °C for 30 sec, annealing at 68 °C for 30 sec, and extension at 72 °C for 1 min with one cycle of a final extension at 72 °C for 5 min. PCR products were run to electrophoresis in 1% (w/v) agarose gel, expected DNA amplicons were cut and purified with GeneJet agarose gel extraction kit (Cat. No. K0691, Thermo). The purified DNA fragments were cloned into pGEM-T Easy vector (Promega, USA). Selected recombinant plasmids containing tritin gene from white bacterial colonies were purified by GeneJet Plasmid Miniprep Kit (Cat. No. K0503, Thermo), then sequenced and analyzed.

2.3. *In silico* Analyses

The tritin gene sequence isolated from the Kutluk-94 wheat cultivar was investigated in the BLASTn database. Expasy's ProtParam online server used to detect amino acid content, charged residue, and molecular weight of Kutluk-94 tritin protein (http://us.expasy.org/tools/protparam.html) (Gasteiger et al., 2005). Various RIP gene sequences from wheat (*Triticum aestivum*), maize (*Zea mays*), barley (*Hordeum vulgare*), fat hen (*Chenepodium album*), great bougainvillea (*Bougainvillea spectabilis*), pokeweed (*Phytolacca insularis*), bitter melon (*Momordica charantia*) and edible amaranth (*Amaranthus tricolor*) were retrieved from NCBI. Using conserved domain architecture retrieval tool (CDART) (https://www.ncbi.nlm.nih.gov/Structure/lexington/docs/cdart_about.html), identification of conserved domains within RIPs was carried out. After the tritin gene sequence (D13795.1) from NCBI database was referenced for the BLASTn search, the presence of tritin genes in barley (*H. vulgare* r1), maize (*Z. mays* PHJ40 v1.2), purple false brome (*B. distachyon* v3.1), intermediate wheatgrass (*T. intermedium* v2.1), resurrection grass (*O. thomaeum* v1.0), yellowwood (*P. latifolius* v1.1), and rice (*O. sativa* v7.0) genomes was screened using the Phytosome v13 database (https://phytozome-next.jgi.doe.gov/). Multiple sequence alignment of different RIPs was performed using the PARALINE Multiple Sequence Alignment (www.ibi.vu.nl). The gene structure of RIP genes was searched using Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/). Phylogenetic analysis of the RIP sequences was performed with Molecular Evolutionary Genetic Analysis (MEGA) software Version 6 by using the UPGMA method.

3. Results and Discussion

The RT-PCR result showed that only one specific DNA band of ~830 bp in length was illustrated in 1% agarose gel electrophoresis (Figure 2). After tritin gene cloning in the pGEM-T-Easy vector, purified recombinant plasmids were bidirectional sequenced. According to the BLASTn result, our tritin sequence showed that tritin gene of size 834 bp shared 94 % identity with tritin sequence of *T. aestivum* from NCBI (D13795.1) (Figure 3). Tritin gene of Kutluk-94 wheat cultivar (K-tritin) has 6 bp nucleotide insertion with GACGGT not found in other tritin sequences. The tritin gene was translated into amino acid sequence and has an initiation amino acid methionine (ATG), and terminated by lysine amino acid (AAA). The K-tritin gene compose of a complete open reading frame and one exon. In BLASTn, tested nucleotide sequence showed 99.40% homology with XM037580949.1, and 93.91% homology with XM037588235.1 and AK330997.1.

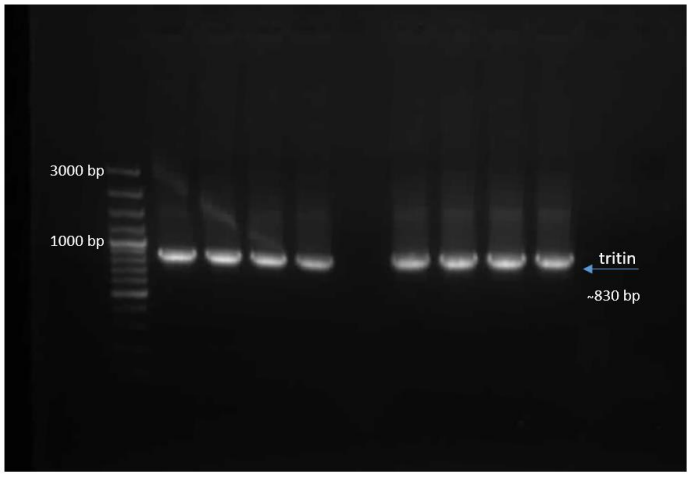

Figure 2. Agarose gel image after PCR amplification of the tritin gene

The amino acid composition of K-tritin as analyzed by the ProtParam online tool was determined to be: mainly 28 Leu

(10.1%), 27 Ala (9.7%), 26 Thr (9.4%), 25 Gly (9%), and 20 Lys (7.2%). The total number of negatively charged residues (Asp + Glu) and of positively charged residues (Arg + Lys) of K-Tritin were 22 and 32, respectively. The molecular weight of the protein was predicted to be approximately 29.9 kDa. Habuka et al., (1993) illustrated that native and recombinant tritin protein was approximately 30 kDa consistent with our result calculated by ProtPram.

Analyses of the conserved region using the CDART online tool revealed that K-tritin shared similar conserved domains with other RIPs. As result CDART, RIP of *Amaranthus tricolor* has not conserved domain. Also, the RIP of *M. charantia* possesses the Ricin-B lectin domain aside from the RIP domain (Figure 4).

T. aestivum tritin gene sequence was used for Blast search in the Phytozome database to detect the presence of tritin protein in other monocot genomes. The numbers of identified tritin-like sequences varied from 1 in *Pharus latifolius* to 18 in *Thinopyrum intermedium*. When compared to other species, *T. intermedium* has a higher sequence similarity with 95% in terms of tritin sequence. In most cases, it was determined that the similarity in tritin sequences of other monocot species ranges from %66 to %95 (Table 1).

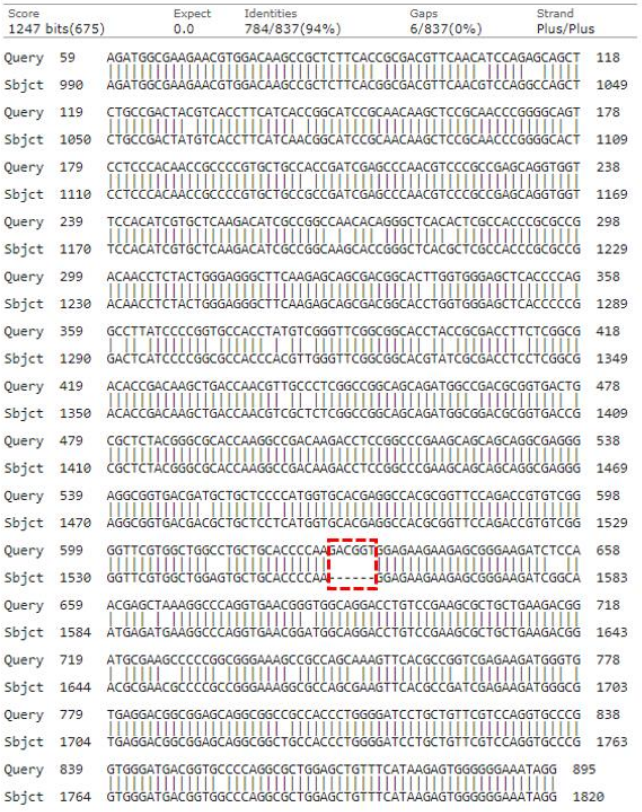

Figure 3. The cDNA sequence of ORF encoding K-tritin and comparison with tritin cDNA (D13795.1). Red box is shown 6 bp insertion in K-Tritin.

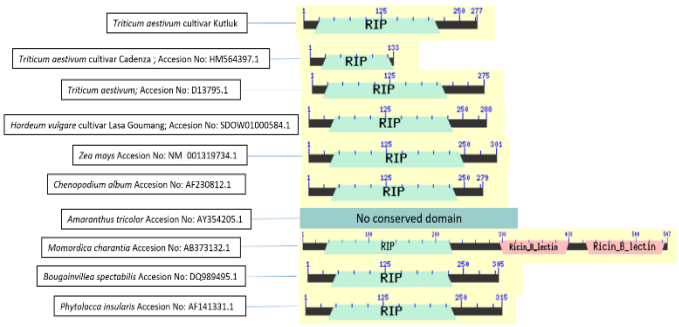

Figure 4. Conserved domains of tritin protein and different Type I RIP

The result of multiple sequence alignment of RIPs showed that K-tritin amino acid sequences were highly similar to amino acid sequences of HM564397.1, D13795.1, SDOW01000584.1, and NM_001319734.1 from NCBI. Although the amino acid sequence of K-tritin is mostly not similar to other RIPs, all RIPs appear to have conserved amino acids such as 33F (phenylalanine), 44Y (tyrosine), 52R (arginine), 105T (threonine), and 182G (glycine). Habuka et al., (1990) illustrated that Tyr-83, Tyr-ll4, Glu-171, Arg-174, and Trp-207 have an important role in RNA N-glycosidase activity. These sequences conserved in cereals are shown with red-dashes boxes in Figure 5. Fabbrini et al. (2017) revealed that these amino acids, which are important for catalytic activity, conserved in several RIPs including momarcharin, bouganin, and PAP.

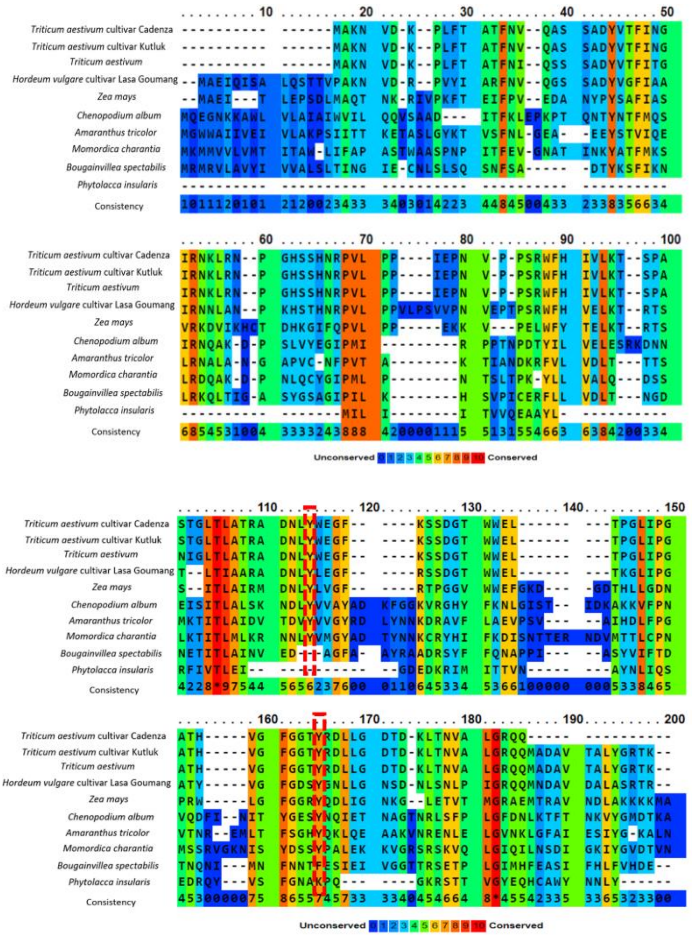

Figure 5. Multiple sequence alignment of amino acid sequences of K-tritin and other RIPs. Red-dashes boxes emphasize active site residues identified for RIPs.

Gene structure of K-tritin and other RIPs evaluated in the current study are shown in Figure 6. According to the Gene Structure Display Server (GDSD) results, there are no introns in the RIPs studied. In addition, the length of the regions encoding the gene and of the upstream/downstream regions vary. Juan et al., (2003) reported that introns were typically an absence of other RIP genes. In our study, K-tritin sequence was not contained introns as previously reported by Habuka et al. (1993).

Table 1. Genomic features and number of sequences matching with tritin (D11) in several monocots by Phytozome v13.

Species	Location	% Identity	Align length	Strands	Target from	Target to
T. intermedium	Chr13	95	834	+/+	366240105	366240938
T. intermedium	Chr13	95	423	+/+	366216808	366217230
T. intermedium	Chr13	75	780	+/-	366230752	366229980
T. intermedium	Chr15	94	834	+/-	532821575	532820742
T. intermedium	Chr15	93	841	+/-	532793866	532793026
T. intermedium	Chr15	94	834	+/-	532871995	532871162
T. intermedium	Chr15	75	830	+/+	532817443	532818263
T. intermedium	Chr15	75	830	+/+	532859901	532860721
T. intermedium	Chr15	84	540	+/+	38126972	38127496
T. intermedium	Chr15	74	463	+/+	532866572	532867034
T. intermedium	Chr15	85	463	+/+	38126595	38126866
T. intermedium	Chr14	91	745	+/+	418899390	418900134
T. intermedium	Chr14	79	639	+/-	418858760	418858138
T. intermedium	Chr14	80	135	+/-	418858056	418857923
T. intermedium	Chr14	88	49	+/+	418900137	418900185
T. intermedium	Chr10	69	160	+/+	273127	273274
T. intermedium	Chr10	69	160	+/-	488147635	488147488
T. intermedium	Chr18	70	133	+/+	3206638	3206758
Hordeum vulgare	Chr5	93	834	+/-	639842331	639841498
Hordeum vulgare	Chr5	92	824	+/-	639669885	639669062
Hordeum vulgare	Chr5	91	834	+/+	639808272	639809105
Hordeum vulgare	Chr5	78	844	+/-	624751436	624750614
Hordeum vulgare	Chr5	87	60	+/-	624738336	624738277
Hordeum vulgare	Chr7	92	834	+/+	226285329	226286162
Hordeum vulgare	Chr7	94	47	+/+	226288493	226288539
O. sativa	Chr1	75	813	+/+	3445453	3446243
O. sativa	Chr1	75	651	+/-	3190245	3189607
O. sativa	Chr1	76	106	+/+	33237162	33237264
O. sativa	Chr1	80	71	+/-	3189485	3189415
O. sativa	Chr1	77	86	+/-	3204581	3204499
O. sativa	Chr12	74	239	+/+	21200708	21200943
B. distachyon	Chr1	74	823	+/-	63158569	63157761
Zea mays	Chr8	67	244	+/-	97176845	97176614
Zea mays	Chr7	69	159	+/+	146829487	146829633
O. thomaeum	Chr2	75	97	+/-	19816	19724
O. thomaeum	Chr2	80	51	+/+	41372	41422
O. thomaeum	Chr2	70	142	+/-	33414	33286
P. latifolius	Chr5	66	587	+/+	68707497	68708047

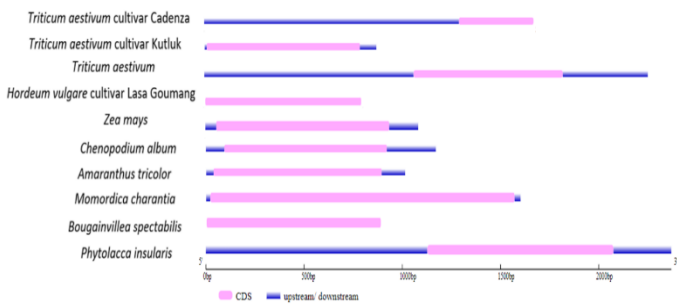

Figure 6. Structure of several RIP genes

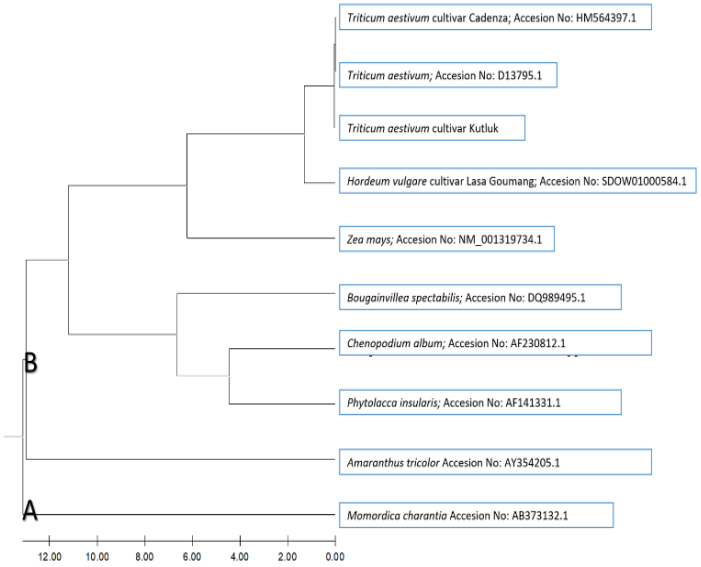

Figure 7. Phylogenetic tree revealing the relationship of K-tritin to different RIPs by Neighbor-joining method

The phylogenetic tree revealed that K-tritin and other tritin (HM564397.1, D13795.1, SDOW01000584.1) were grouped together (Figure 7). The phylogenetic tree is divided into two main groups, A and B. Type I RIPs clustered to B group, whereas type II RIP from *M. charantia* is assigned to A group. Girbes et al., (2004) reported that almost 36 plant RIP genes have been characterized and their protein sequences are present, but there is very little information about genome structure and organization for RIPs of these species.

4. Conclusion

Recently, studies on RIPs have increased due to their potential use in treatment of diseases such as cancer (Allahyari et al., 2017), AIDS (Hogan et al., 2018), and autoimmune diseases (Benitez et al., 2005). RIPs have been used in plant defense due to antifungal, antiviral, and antibacterial activities (Madin et al 2000, 8; Donayre Torres et al 2009, Abbas 2007, Kim et al., 2003, Güller et al., 2016). In this study, the sequence of K-tritin was evaluated using different bioinformatics tools and compared with several RIPs. Although the biological, molecular, and structural properties of many RIPs have been reported previously, the literature on tritin is very limited. Therefore, the outputs of the present study contribute to this inadequacy in the literature.

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