Advances in Bioscience and Biotechnology, 2016, 7, 531-538
http://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Structure of Alpha-Gliadin Multigene and Construction of Efficient Hairpin RNAi Molecule against Gluten Enteropathy

Saeedeh Sadegzadeh*, Bahram Baghban Kohnehrouz

Department of Molecular Biology and Biotechnology, University of Tabriz, Tabriz, Iran
Email: *Saideh.sadegzadeh@gmail.com

How to cite this paper: Sadegzadeh, S. and Kohnehrouz, B.B. (2016) Structure of Alpha-Gliadin Multigene and Construction of Efficient Hairpin RNAi Molecule against Gluten Enteropathy. *Advances in Bioscience and Biotechnology*, **7**, 531-538.
http://dx.doi.org/10.4236/abb.2016.712047

Received: October 24, 2016
Accepted: December 4, 2016
Published: December 7, 2016

Abstract

Celiac disease is known as gluten enteropathy, caused by damaging of small intestinal epithelium cells following gluten consumption. Gluten is classified in two families of glutenin and gliadin. Gliadin is divided into α, γ and ω groups. One of the most effective methods to rectify or minimize gene expression is RNAi technology. The present study has been conducted to the structure of α-gliadin gene to produce proprietary RNAi cassette for silencing wheat α-gliadin gene. So nucleotide sequence of α-gliadin gene involved in design for PCR amplification by online primer-blast software. The amplification was extracted from agarose gel and ligated to pTG19 cloning vector. After cloning of the recombinant plasmid in *E. coli*, they were sequenced. Then they were used for construction of specific and efficient RNAi cassette. The produced vector by this strategy is considered as an effective step in developing genetically engineered gluten-less or low content gluten wheat.

Keywords

Cloning, Gluten, RNAi Technology, Vector, Wheat

1. Introduction

Celiac disease is also known as gluten enteropathy, and known as gluten intolerance disease. Celiac disease is an inherited disorder of the immune system that damages the lining of the small intestine, caused by gluten consumption and may impair the absorption of nutrients. People with celiac disease couldn't to endure a protein in wheat, barley, rye, and oats, called gluten. Today, the only way to treat celiac disease is gluten-free diets that all foods contain gluten are removed. Several studies have been conducted in this connection in order to produce gluten-free or very low gluten content product.

Gluten is classified in two families of glutenins and gliadin [1]. Gliadins are divided to three structural classes of α, γ and ω groups [2]. However, α-gliadin components are most important allergens [3]. Number of copies of hexaploid wheat α-gliadin encoding gene is estimated at about 25 to 150 copies [1]. One of the effective methods for controlling allergen gens expression is RNAi technology (RNA interference) which used to extinguish or reduce gene expression. This method is now widely used for gen silencing in plants. RNAi is a conserved mechanism in a wide range of eukaryotic organisms except *Saccharomyces cerevisiae* [4]. RNAi inactivates gene expression in a sequence-specific manner [5]. In contrast, RNAi in plants is usually established by transformation with a construct that produces hairpin RNAs [6]. RNAi vector consists of an inverted repeat harbouring target sequences under the control of a strong promoter. The inverted repeat sequences are separated by a spacer fragment [5]. RNAi technology is effective and flexible in gene silencing in plants that led to the widespread use of this technology. The purpose of this research is producing proprietary RNAi cassette for wheat α-gliadin. This plasmid is used for silencing specific genes and provides a means to reduce the gluten.

2. Materials and Methods

2.1. Materials

Triticum durum L. cv. Aria seeds provided from Karaj Plant Breeding department. *Escherichia coli* strain DH5α (Invitrogen, USA) was used for cloning. The sequencing vector pTG19, Taq DNA polymerase and restriction enzymes were purchased Vivantis (USA) and Fermentas Corporation respectively. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Thermo Fisher Scientific Inc. (USA). DNA gel extraction and plasmid extraction kits were purchased from Bioneer Corporation (South Korea). Sequencing was conducted by BIONEER Co. (South Korea).

2.2. Methods

2.2.1. Genomic DNA Extraction

Seeds were cultivated under standard conditions in a green house. After growing in 2 - 3 leaves stage, leaf tissue genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB). Extraction method as described by Saghai-Maroof [7]. Its quality and quantity were determined by 0.8% agarose gel electrophoresis.

2.2.2. Bioinformatics' Analysis and First Step of Primer Design

The sequences of α-gliadin gene from *Triticum spelta* (GeneBank accession number AJ130948.1) were retrieved from the NCBI Gene Bank at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). In order to design specific primers, CLUSTALW program was used to carry out nucleotide sequence alignments. Forward Primer 5'ATCCATGGCCAATTTCACAGCAGCAGCA3' and reverse primer 5'ATCTGCAGAGGCTGTTGGAAGGAGACC3' were designed using Primer

3 software at NCBI and their validity were confirmed by Oligo 7 software. The restriction enzyme sites, NcoI and EcoRI were added to 5'-ends of FP and RP primers.

2.2.3. PCR Amplification and Cloning

The total genomic DNA was used as a template for the amplification of *α*-gliadin Using RF and RP primers. The reaction was carried out in a total volume 25 microliter (µl). The amplification program was done as the primary denaturing using 94˚C for 5 minute (min), followed by 35 cycles of 94˚C for 50 second (sec), 60˚C for 45 sec, and 72˚C for 2 min, and a final extension step at 72˚C for 7 min. The amplified DNA products were separated by 0.8% agarose gel contains Red Safe and extracted using DNA gel extraction kit (Bioneer Co., Cat. No. 1/3035). The purified product were ligated into pTG19 vector (Takara Co.) and transformed into competent cells of *E. coli* DH5*α* strain. Recombinant colonies were selected by white-blue test on Luria-Bertani (LB) medium containing antibiotic ampicillin (100 µg/ml), IPTG, (100 µg/ml) and X-gal (1 mM) (Figure 1). The positive white colonies plasmid was extracted by Plasmid Extraction Kit (BIONNER Co.). Desired fragments were sequenced by the BIONEER Company (South Korea).

2.2.4. The Second Step of Primer Design

The obtained sequence was analysed by BLAST software. The specific new forward primer 5'ATCCATGGTGCAGCAACACAACATAGCG3' and reverse primer 5'ATGAATTCGAGGCTGTTGG AAGGAGACC3' designed to conduct for synthesis of target sequences from desired genes. NcoI and EcoRI restriction sites were added to 5'-ends of Forward and Reverse new primers.

2.2.5. Create the RNAi Vector

After verification, the ligation reaction was performed between two fragments to create final construction. The two-step procedure was carried out to making alpha gliadin RNAi cassette. Product I (481 bp fragment) followed by directional sub-cloning of its

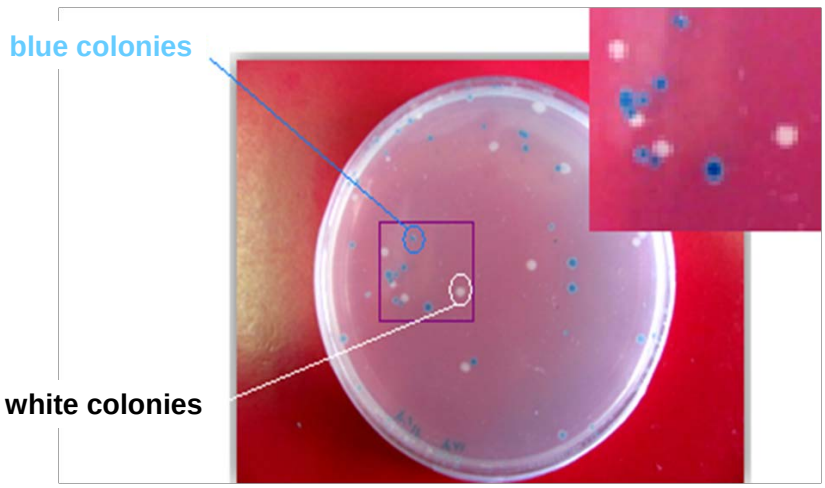


Figure 1. Bacterial colonies transformed with pTG19 vector.

inverted orientation shorter fragment (product II, 331 bp). These two inverted regions separate by a 133 bp nucleotides as a spacer fragment (Figure 2(a)). Plasmid extraction was performed (using Bionner extraction kit) from liquid culture of white colonies after confirming by colony PCR method (Figure 3(b)). Purified plasmid DNA was sent to sequencing.

3. Results and Discussion

3.1. Structural Feature of Designed RNAi

Expression of this cassette by an appropriate promoter produce RNA molecule consisting of two inverted repeat fragments, leading to a hairpin structure. These two inverted

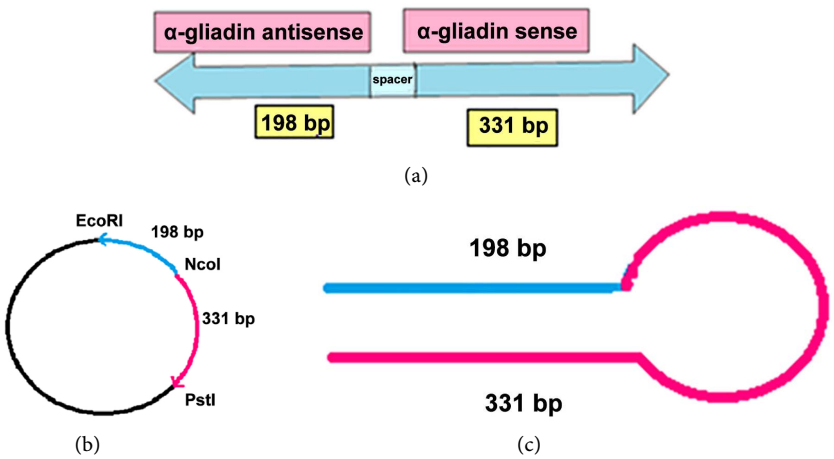


Figure 2. Fragment's position and RNAi schematic design. (a) Position and the length of sense and antisense amplified fragment, product (I) and product (II); (b) Position of fragments in the vector and restriction used enzymes; (c) Schematic structure of hairpin and the stem section.

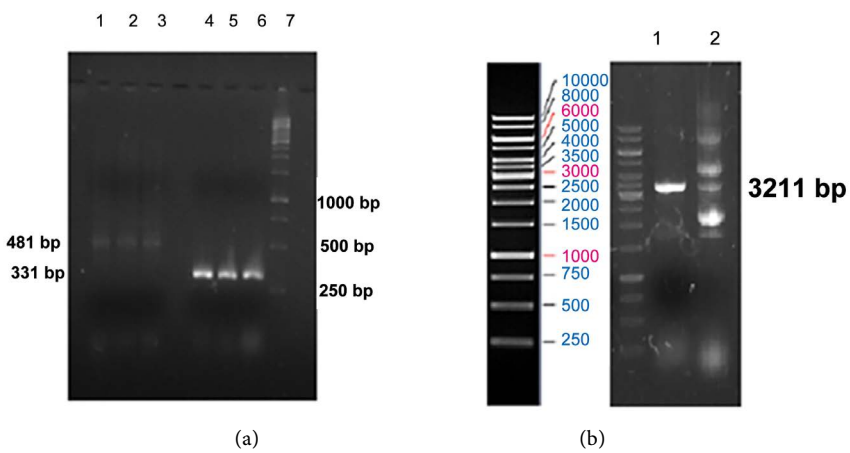


Figure 3. Identification of target fragments. (a) 1, 2, 3: PCR amplification of alpha gliadin 481 bp fragment (produt 1), 4, 5, 6: PCR amplification of alpha gliadin 331 bp fragment (product 2), 7: The DNA Marker; (b) Construction and identification of designed RNAi, 1: Recombinant plasmid, 2: Recombinant plasmid digestion with NcoI.

regions separate by a 133 bp nucleotides as a spacer fragment. The stem section of this construct contains about 198 bp out of 331 bp nucleotides as a double strand RNA. This region is a substrate for dicer and would produce small interfering RNA molecule to be anial to *α*-gliadin mRNAs as well as a primer to double stranding mRNAs by RNA-dependent RNA polymerase (RDRP) enzyme (**Figure 2(c)**).

3.2. Alpha Gliadin Gene Structure

Alpha gliadin gene protein sequence had a common structure model: a short signal peptide with 20 amino acid residues followed by 5 distinct domains: a repetitive domain containing N-terminal With 5 amino acid residues in the beginning a polyglutamine domain I, a unique domain I, a polyglutamine domain II and a unique domain II containing C-terminal in the end (**Figure 4**).

3.3. Nucleotides and Amino Acids Sequence Comparison

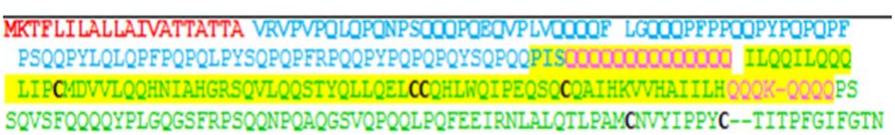
3.3.1. Comparison and Sequencing Analysis of Amplified *α*-Gliadin Fragment

The Results of sequencing of *Triticum durum* L. cv. Aria seed *α*-gliadin cloned fragment was compared with other 2547 alpha gliadin genes sequences from 19 different *Triticum* species reported in NCBI GenBank (**Table 1**). Nucleotides sequence comparison was based on multiple alignments by BLAST software. The nucleotide alignment results validated the sequence data of alpha gliadin with high homology identities more than 90%. The results showed that Aria alpha gliadin gen had high similarity with other different species. Therefore the designed RNAi structure to alpha-gliadin gene silencing can be used in a wide variety of gramineae.

3.3.2. Comparison of *α*-Gliadin Gene Molecular Characterization

Triticum durum L. cv. Aria seed *α* -gliadin gene was used to detect amino acid sequences. Prediction of deduced amino acid sequences was carried out by COBALT software and compares ypi with 11 other alpha-gliadin amino acid sequences from 11 species reported in NCBI GenBank (**Figure 5**). Amino acid sequence comparison demonstrated that this cloned gene possessed tcal structural features of previously characterized *α*-gliadin genes. The multiple alignment of deduced amino acid sequence showed that the Aria *α*-gliadin gene had highly similar structure with other register

(a)



(b)

Figure 4. The structure of alpha gliadin gene. (a) Gene common structure; (b) Amino acid sequence and cloned fragment region (The region is highlighted in yellow).

Table 1. Content of alpha gliadin genes in *Triticum* species, a comparative analysis and alignment result between Aria seed alpha gliadin gene and registered *α*-gliadin gene sequences in *Triticum* species.

Total alpha gliadin genes in *Triticum* species Total: 800			
Name	**Number of registered *α*-gliadin genes**	**Name**	**Number of registered *α*-gliadin genes**
Triticum aestivum	333	*T. sphaerococum*	16
Triticum monocccm	89	*T. turgidum* sp. *poleacolchicum*	9
Aegilops tauschii	77	*T. turgidum* subsp. *turgidum*	6
Triticum urartu	57	*T. polonicum*	5
Triticum dicoccoides	57	*T. monococum* subsp. *armeniacum*	3
T. Compactum	51	*T. spelta*	3
Aegilops speltoides	37	*T. macha*	2
Thinopyrum ponticum × *T. aestivum*	27	*T. spelta var arduini*	1

Name	**Total alignment**	**Number**	**Alignment result**	**Identic**	**Query cover**
T. aestivum					
T. monococcum	100	96	*α*-gliadin isolate A3 avenin-like protein gene	90%	95%
		4	complete cds	75%	23%
Aegilops tauschii	100	100	*α*-gliadin	93%	92%
Aegilops speltoides	55	48	*α*-gliadin *Aegilops speltoides* isolate A10 avenin	90%	95%
		3	like protein *A. peltoides* mitochondrial DNA	73%	27%
		1	complete cds *A. speltoides* isolate spE0061	86%	10%
		1	chloroplast complete gene *A. speltoides* (Spa)	100%	4%
		2	clone BAC	94%	5%
T. urartu	66	59	*α*-gliadin	92%	95%
		6	T. urartu clone BAC complete cds	94%	5%
		1	T. urartu chloroplast complete sequence	86%	6%
T. dicoccoides	60	58	*α*-gliadin *T. dicoccoides* clone BAC genomic	93%	95%
		2	sequence	90%	6%
T. compactum	41	41	*α*-gliadin	97%	77%
T. turgidum subsp. *durum*	38	25	*α*-gliadin *T. turgidum* isolate A avenin like protein	91%	95%
		6	psedogen complete sequence	73%	27%
		7	*T. turgidum* clone BAC	90%	5%
T. turgidum subsp. *poleacolchicum*	14	9	*α*-gliadin *T. poleacolchicum* Ppd-B1 gene for	92%	95%
		5	Pseudo response regulator	100%	3%
T. turgidum subsp. *turgidum*	6	6	*α*-gliadin	86%	13%
T. polonicum	5	5	*α*-gliadin	91%	95%
T. timophevii subsp. *armeniacum*	2	2	*α*-gliadin	90%	95%
T. aestivum subsp. *macha*	3	3	*α*-gliadin	86%	93%
T. sephaerocum	19	16	*α*-gliadin spelt factor protein MADS-box transcription factor (MADS) gene	91%	95%
		2		93%	4%
		1		100%	3%
T. spelta	3	3	*α*-gliadin	93%	93%
T. spelta var arduini	1	1	*α*-gliadin	93%	92%
T. zhakovski	3	1	*α*-gliadin	90%	91%
		1	LMW	100%	10%
		1	(Matk) gene	93%	4%

Figure 5. Representative protein accetion numbers and multiple alignment of the deduced amino acid sequences of Aria alpha-gliadin genes with amino acid sequences of 11 different *Triticum* species (Red region: signal Peptide, blue region: N-terminal repetitive domain, violet regions: poly glutamine domain I and II, gr).

α-gliadin genes in the GenBank. The produced results and comparison between the cloned sequence and other species, confirmed that the main allergen region in alpha

gliadin gene had been detected properly and selected for design a specific and efficient hairpin RNAi molecule against gluten enteropathy.

4. Discussion

Alpha-gliadin is the most abundant wheat seed protein that has basic and principle role in the sensitivity of patients with Celiac disease. RNAi technologies make it possible to effectively down-regulate the target mRNA of alpha-gliadin multigenes from wheat, which causes celiac disease symptoms in genetically predisposed individuals. Thus, we have designed and developed a specific and an efficient RNAi cassette as a critical step for targeting wheat alpha-gliadin. According to our results, it appears that with respect to the selected sequence and final design of its structure, it is an efficient and suitable one for controlling the allergen genes and in particular alpha-gliadin gene family in all groups. This would be in wheat and in all variety of gramineae to create an effective silencing gene.

Acknowledgements

The authors are grateful for support of this work by the University of Tabriz.

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