Designing highly effective and genetically invert fragments by full assessment of mutations in seed region siRNAs in omega gliadin epitopes

Mohammad Ali Ebrahimi[1*], Mahdi Azari-Anpar[1], Nassim Zarinpanjeh[2]

[1]Department of Agricultural Biotechnology, Payame Noor University, P. O. Box: 19395-3697, Tehran, Iran.
[2]Department of Biotechnology, Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.
[*]Corresponding author, Email: ma_ebrahimi@pnu.ac.ir. Tel: +98-2122455001. Fax: +98-2122455106.

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Abstract

RNAi mechanism plays a major role in silencing the expression of target genes by siRNAs. In the current study, *in silico* properties of 30 genes in omega-2 gliadin and 266 nt and 326 nt mutations were investigated before and after cloning in an expression vector. Specific primers were designed for 30 genes with spacer regions of 75 nt and 178 nt (for gene invert repeats). The frequency of siRNA site and nucleotide mutations A/U to G/C were identified for target mRNA using *in silico* analysis. The results showed that invert repeat sequences consisting of spacer region of 178 nt had a high efficiency for target gene without nucleotide mismatches from A/U to G/C. Spacer regions were designed with long fragments for RNAi cassette instead of intron sequences. Paying enough attention to observe the location of mismatch nucleotides in siRNA and length spacer region before and after cloning reduced the deletion time of intron. At the same time, removal of epitopes in wheat dependent exercise-induced anaphylaxis (WDEIA) and celiac disease could be considered as advantages of the applied method compared to ligation in the bacterial vector.

Key words: Celiac disease, Off-targeting, Omega gliadin, RNAi, siRNA.

INTRODUCTION

Omega gliadin proteins are one of the subunits of wheat gluten and are classified into two sections of slow and fast omega gliadins, based on their molecular weight. Fast omega gliadins are also called omega-5 gliadins (Matsuo *et al.,* 2004) or lighter omega gliadins (Waga *et al.,* 2004) which are genes encoded by chromosome 1B, at the Gli-B1 locus (Anderson *et al.,* 2009). Also slow omega gliadins (Matsuo *et al.,* 2004) or *omega-2 gliadins* (Wieser, 2007) are encoded by genes on the short arms of chromosome 1D (Matsuo *et al.,* 2004; Wang *et al.,* 2008). Omega gliadins are repeated sequences that contain L-glutamine and L-proline amino acids. In omega gliadin protein, glutamines form more than 45%–55% of *omega-2 gliadin* and *omega-5 gliadin*, respectively (Wieser, 2007). In omega gliadin, the CAA/CAG ratio of glutamine codons in three wheat genomes ABD is around 7:1 (Anderson *et al*., 2009). These glutamines (poly glutamines) are the main causes of food allergy known as wheat dependent exercise-induced food anaphylaxis (WDEIA) and celiac disease (Ensari *et al*., 1998; Matsuo *et al*., 2004). Both omega gliadins contain food allergy associated with WDEIA. In poly glutamine omega-5 and *omega-2 gliadin,* the indicated peptide sequence i.e. fast omega gliadin proteins are found more in celiac than in WDEIA (Matsuo *et al.,* 2004). Epitopes inducing *omega-2 gliadin* in WDEIA and celiac diseases are QPQQPFP and PQQPFPQQ, respectively (Ensari *et al*., 1998; Battais *et al*., 2005). Novel wheat lines with altered gluten protein compositions can be used for patients of WDEIA with the help of ribonucleic acid interference (RNAi) technology (Altenbach *et al*., 2013).

RNAi strategy is triggered by hairpin RNA (hpRNAi). Vector structure has been widely used as one of the most efficient tools to explore gene function. This technology is the best method to alter the gene expression in plants for improvement in quality traits. There are several methods for the construction of RNAi cassette like isothermal *in vitro* recombination system. Among them, there are certain steps that are commonly being practiced. These include: I- Identification of target gene using genome sequencing by applying bioinformatic tools and also analysis of transcriptome, proteome and metabolome; II- Selecting suitable vectors and promoters along with screening by implementing selectable markers, which play a major role for making RNAi constructs and screening RNAi cassette (Jiang *et al*., 2013). This technology has been successfully used in other wheat gliadins, 14-16 kD proteins in rice and other crops (Tada *et al*., 1996; Gil-Humanes *et al*., 2010). The unifying features of ribonucleic acid silencing (or RNAi) phenomena are production of short (21–26 nt) RNAs that act mainly as specificity determinants for reducing gene expression (Tang *et al*., 2003). Two active enzymes called DICER and RISC, after delivery of RNAi cassette in *in vivo* cells, are the main causes of reduction of gene expression (Saurabh *et al*., 2014).

Short double-stranded RNA or small RNAs are generated in animal cells by members of the Dicer family of long double stranded RNA (dsRNA)-specific endonucleases, and in plant cells by an RNaseIII-Like enzyme called Dicer Like 4 (DCL4) (Tang *et al*., 2003; Senthil-Kumar and Mysore, 2014). Next, small interfering RNAs or short double-stranded RNAs are incorporated into endoribonuclease-containing complexes known as RNA-induced silencing complex (RISC) in which small interfering RNAs (siRNAs) play a major and guiding role in sequence-specific cleavage of target mRNAs. This can occur successfully in different stages during post-transcriptional gene silencing (PTGS) in plant cytoplasm and other eukaryote genomes. The siRNAs are sequences of 21 nucleotides (nt) which are composed of three different regions including seed region, central region and 3′ region. Single-nucleotide mismatches in the seed region have the greatest impact on reducing on-targeting and increasing off-targeting for target gene silencing. In total, siRNAs are made of double-stranded sense (passenger) and anti-sense (guide) in which single stranded anti-sense is considered as a major tool playing a significant role in PTGS. Cleavage and destruction of the target mRNA take place almost in the middle of the region bound by the guide siRNA strand (Senthil-Kumar and Mysore, 2014). In this study, we describe various parameters involved in identifying the best situation for siRNAs in fragments of sense-antisense and comparison of hairpin RNAi cassettes with different lengths for sequence of epitopes CD and WDIEA with no need to use fragment intron in making cassette RNAi after sequencing.

MATERIALS AND METHODS

Plant material and DNA extraction

Wheat (*Triticum aestivum subsp. aestivum*; 2n=6x=42; ABD genome) seeds of synthetic variety of AZAR2 (developed in Iran, Jihad-e-Agriculture. of Ahar) were planted in pots (leaf soil+clay). Plants were grown at the constant temperature about 25–26 ºC with a 16L/8D photoperiod for 18 days. Leaf samples at 2–3 leaf stage were ground in liquid nitrogen and subsequently total genomic DNA was extracted by conducting a CTAB method (Murray and Thompson, 1980). Quality, quantity and concentration of the extracted target genomic DNA were evaluated by 1.6% agarose gel electrophoresis using 1kb DNA size marker (SinaClon Co., Tehran, Iran).

Cloning fragments into vector

The T/A cloning vector, pTG19-T (Vivantis Co., USA), and expression vector, pAHC25 (constructed by Christensen and Quail, 1996) were used for cloning PCR products and the invert fragments, respectively. The *omega-2 gliadin* genes (326, 249, 266 and 191 bp) of *T. aestivum* were cloned in the pTG19-T vector or the purified (by gel extraction kit, Bioneer Co, South Korea) PCR products of the *omega-2 gliadin* were used as a template for amplifying the stems and caps of RNAi. After purification of the fragments of invert repeats (326 bp+249 bp and 266 bp+191 bp), they were ligated into the pAHC25 binary vector.

Identification of siRNAs

An siRNA data set (21 nt) was analyzed in our study for comparison of two invert fragments with different lengths. This data set contains the sequences and knockdown data for 6 siRNA sequences selected from the transcript sequence positions. The siRNA sequences were evaluated for *moega-2 gliadin* gene, after conversion of DNA to RNA (Markham and Zuker, 2008) for two fragments of 326 and 266 bp. RNA secondary structure stability with the 2 nt RNA dangling at the 3′ end, represented by ΔG value, was calculated with unafold software (Markham and Zuker, 2008). Evaluation of mismatches in single-nucleotides were carried out in anti-sense strand for target mRNA after automated sequencing facilities (Bioneer, South

Table 1. The primer sequences, length, Tm and G/C of FPn* and RPn*.

Sequence (5'→3')	Length (bp)	Tm (°C)	Enzyme sites
FP1 AG**GCTAGC**AACCATATCCACAACAACCAT	30	61.9	*Nhe*I
RP2 AG**cccgggCCATGG**GATTGTTGGGTCTGGGGAAA	34	62.0	*Nco*I and *Sma*I
F1/R2 Product	266	87.3	-
FP3 AG**GCTAGC**AACCATTTCCCACACCCCAA	28	58.4	*Nhe*I
RP4 AG**gagctcGAATTC**TGTTGTGGTTGCCAGGGA	32	60.0	*Eco*RI and *Sac*I
F3/R4 Product	191	85.9	-
FP5 AA**gagctc**AAGGGCTACAATCACCTCAA	28	57.5	*Sac*I
RP6 AA**CCATGG**AGAGGGAAAGATTGTTGGG	27	59.0	*Nco*I
F5/R6 Product	326	86.5	-
FP7 AA**cccgggGAATTC**AAAGGGCTACAATCACCTC	33	59.7	*Eco*RI and *Sma*I
RP8 AA**CCATGG**GGTTGTTGGGGTTGCTGGG	27	63.9	*Nco*I
F7/R8 Product	249	85.7	-

*: Numbers of forward and reverse primers for *omega2- gliadin* gene sequence.

Korea). The web server for mismatch design uses an algorithm available in the desiRm software (Ahmed and Raghava, 2011). This online software showed all mismatches in antisense fragments from 3′ end to the 5′ end. The major nucleotides that create mismatches contained A-U-G-C.

Designing the primers

In this study, before designing the required primers, major omega gliadin genes were identified in NCBI. They were related to 1D type *omega-2 gliadin* genes in bread wheat. The ClustalW online software (Thompson *et al.*, 1994) was used to assess 1D type omega gliadin for identifying the best regions for designing primers. The sequence of *T. aestivum omega-2 gliadin* gene (Gene Bank accession number, kf412579) consisting of coding regions of 924 bp *omega-2 gliadin* gene, was retrieved from NCBI in order to design specific primers (Table 1) for the repetitive regions of CAA codon (>50%). CD and WDIEA of 1D type omega gliadin gene were designed using Primer-BLAST online software and eventually their validity was confirmed by Oligo7 tool. The restriction enzyme sites were added to the 5′ end of the forward and reverse primers for further subcloning.

PCR amplification, bacterial transformation and hpRNAi vector construction

The total genomic DNA of bread wheat was used as a template for the amplification of *omega-2 gliadin* genes. The PCR program was set as: the initial denaturing at 94 °C for 5 min, followed by 35 cycles of 95 °C for 60s, 63.5 °C and 60 °C (for fragments of 266 bp and 326 bp, respectively) for 45s and 72 °C for 60s, and a final extension step was performed at 72 °C for 5 minutes. The same program was used for the amplification of 185 bp and 243 bp fragments but the only difference was in annealing temperature (61.5 °C and 64.5 °C, respectively). Two fragments of 266 bp and 326 bp were extracted from the 1.6% agarose gel, using the extraction kit (Bioneer, South Korea Co.). These amplicons were used at the concentration of about 21 ng/µl for ligation into pTG19-T and a molar ratio of 1:3 vector to insert and two temperatures of 4 °C and 20 °C were used for 24 h. The *E. coli* DH5α competent cells were used for heat shock transformation (ice 45 min, water bath 42 °C for 1 min and ice for 3 min). For this purpose, 100 µL of competent cells were used in 4 µL of ligation reaction. The transformed cells were selected on white-blue colonies in the test media; LB agar plates containing ampicillin, X-gal and IPTG (Sambrook and Russell 2001). The recombinant plasmid DNA was extracted from the positive (white) colonies using the extraction kit (Bioneer Ltd. South Korea). The the presence of 266 and 326 bp fragments in pTG19-T was confirmed by *Nco*I digestion. The sequences obtained from the colonies potentially possessing *omega-2 gliadin* were sequenced by automated sequencing (Bioneer, South Korea). For making inverted fragments (Figure 1), sequencing two long fragments plays an important role. In this regard, mismatches were identified using desiRm online software (OLIGO Primer Analysis Software Version 7, Molecular Biology Insights, Inc., Cascade, CO, USA) (Table 1).

The RNAi construction process for short fragments was carried out as follows: colony PCR was performed with FP3/RP4, and FP7/RP8 (Table 1) and PCR products (30–40 ng/µL) were mixed with recombinant plasmid (containing pTG-19 vector and long fragments) with

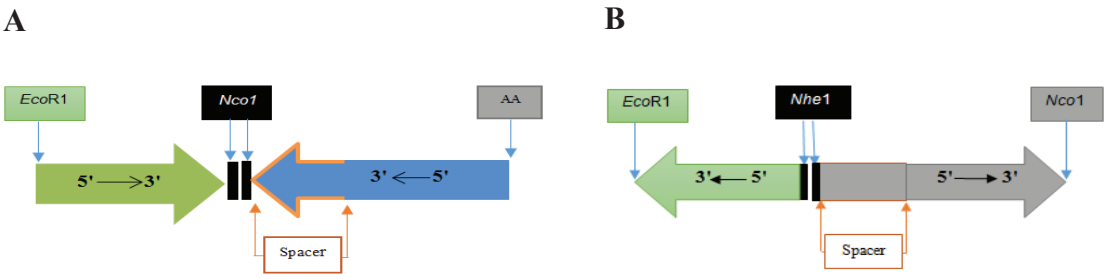

Figure 1. Inverted fragments for making RNAi constructions omega2-gliadin. **A:** Construction of related 563 bp fragment (326 bp+249 bp), **B:** construction of 445 bp (266 bp+191 bp).

Table 2. Oligonucleotides used for RNAi with high efficiency for part of accession number kf412579.

Number	Target position	RNA oligo sequence 21nt anti-sense (5′-3′)	GC (%)	ΔG° (anti-sense and sense)
1	86-108	AUUGUUGAGGUGAUUGUAGCC	40.4	-26.4
2	105-127	UGUUGUUGAUGGGAAAAUGAU	35.7	-27.7
3	112-134	AAAUGGUUGUUGUUGAUGGGA	40.4	-27
4	223-245	AAAUGGUUGUUGUGAUUGCUG	38.0	-28
5	224-246	UAAAUGGUUGUUGUGAUUGCU	35.7	-24.7

1, 2 & 3: Exist in 310 nt fragment only. 4 & 5: Sequences of exist in either 244 nt and 310 nt fragments.

molar ratio of 3 to 1, respectively. After incubation at 22 °C for 1 hour, the product (2 µL) was transferred into *E. coli* DH5α competent cells using the heat shock method (Sambrook and Russell, 2001). After incubation at 37 °C for 1 hour in 700 µL of LB-broth medium, 100 µL of the culture was plated onto LB agar containing 100 mg/L ampicillin. Colonies were then screened by restriction enzyme digestion (Sambrook and Russell, 2001) of a positive colony containing the long and short fragments.

RESULTS

Bioinformatic study

Implementation of bioinformatic software and quick methods are considered important factors in making hpRNAi constructions for the gene fragments encoding poly glutamines. Successful amplification of the long fragments is a major factor in stem and caps of RNAi cassette. This software is suitable for easy identification of single nucleotide DNA in different regions.

ClustalW

Alignment of 30 omega gliadin genes available in NCBI was carried out by ClustalW (Figure 2). This software could identify the best region for primer design from nucleotide sequences between 140 and 383 bp in *omega-2 gliadin* gene associated with GenBank accession number, kf412579.

Identifying siRNA douplexes and mismatches

The initial siRNA was studied with sense and antisense strands of 19 nt and 2 nt (dangling 3′ end) in *omega-2 gliadin* gene. In this study, the most important siRNA based on ΔG value and G/C% (Table 2) was selected. The single-nucleotide mismatches would probably identify antisense strands of the siRNAs (Table 3). They occur in three seed (5′ end), central, and 3′ (3′ end) regions which can change on-targeting to off-targeting.

The single-nucleotide mismatch identification after cloning and sequencing was carried out for the assessment of 266 bp and 326 bp fragments for making highly efficient invert fragments.

Amplification of ID type of omega gliadin

The sense fragments of omega gliadin were then obtained by PCR amplification via primers PF1/RP2 and PF5/PR6. Two 266 nt and 326 nt sequences of wheat from 1D genome was amplified by PCR using

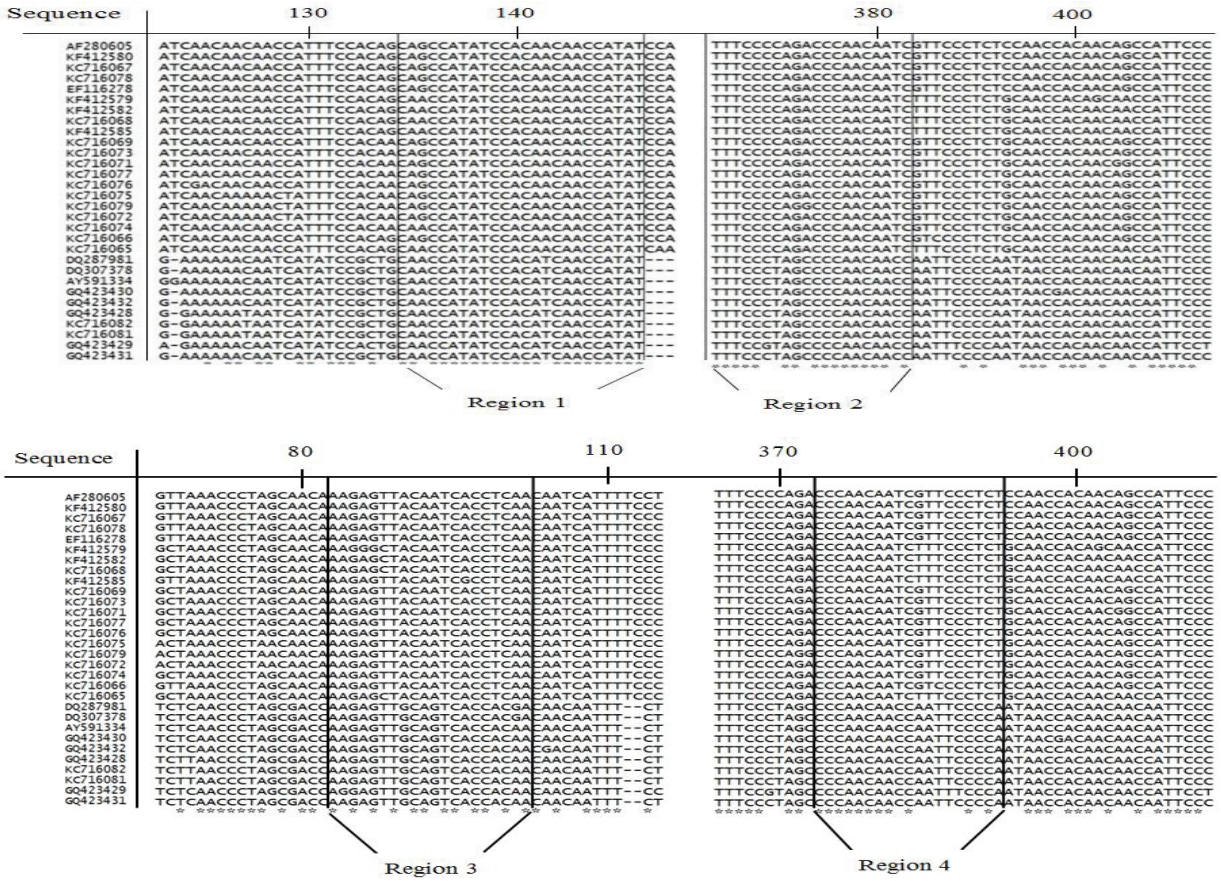

Figure 2. Comparing sequences available in NCBI by Clustalw for omega gliadin genes 1D type. Regions 1 and 2 which selected for 244 bp (without enzyme sites) fragments related to forward and reverse primers and regions 3 and 4 are related to 310 bp (without enzyme sites) fragment used for initial designing of forward and reverse primers.

Table 3. Single-nucleotide mismatches controlling 19 nt 5′ end in different anti-sense regions in omega 2-gliadin.

RNA sequence (5′-3′)	Seed region (%)	A→G/C (%)*	U→G/C (%)*
AUUGUUGAGGUGAUUGUAGCC	36.84	9.52	38.09
UGUUGUUGAUGGGAAAAUGAU	36.84	0	38.09
AAAUGGUUGUUGUUGAUGGGA	36.84	19.04	4.76
AAAUGGUUGUUGUGAUUGCUG	36.84	19.04	28.57
UAAAUGGUUGUUGUGAUUGCU	36.84	28.57	19.04

Red colour: seed region, green colour: central region, blue colour: 3′ region.
*: Calculation for seed region sequence.

specific primers. The results obtained are shown in Figure 3. These sense fragments were ligated into pTG19-T to obtain pT-Ome1D-Whe. The cloned sense fragments in pTG19-T were confirmed by colony PCR, digestion by *Nco*I restriction enzyme and agarose gel electrophoresis (Figure 4). Single digestion with *Nco*I enzyme indicated two fragments of 3146 bp and 320 6 bp containing 2880 bp pTG19-T, and two 266 and 326 fragments of type 1D omega gliadin (Figure 4). By using the gene sequence, epitopes of WDEIA and CD were analyzed using NCBI online software for nucleotides and protein sequences (Figure 5).

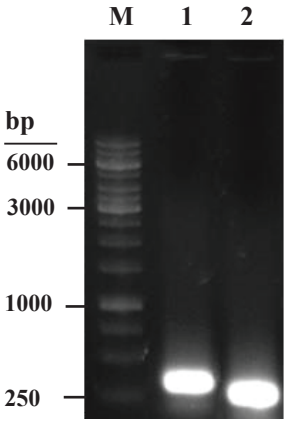

Figure 3. PCR product 1 and 2: fragments 326 bp and 266 bp of type 1D omega gliadin, M: DNA Marker.

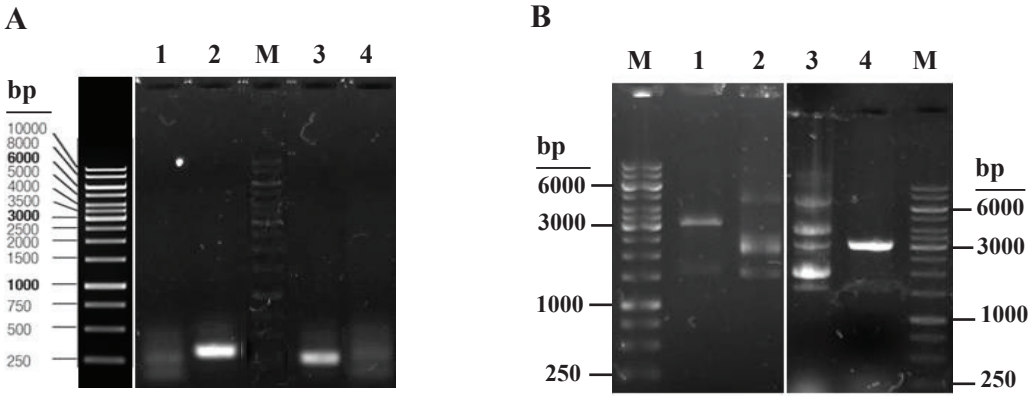

Figure 4. Construction and identification of type 1D omega gliadin fragment. **A:** M: The DNA Marker, 1 and 4: PCR amplification of pT-Ome1D-Whe non recombinant plasmids for fragments 326 bp and 266 bp, 2 and 3: PCR amplification of pT-Ome1D-Whe recombinant plasmids related to sense fragment 326 bp and 266 bp. **B:** M: DNA size marker 1Kb, 1 and 4: digestion product pT-Ome1D-Whe with *NcoI* for fragments of 326 bp and 266 bp, 2 and 3: Uncut plasmid fragments of sense 326 bp and 266 bp.

Table 4. Features of siRNAs and mismatch after cloning and sequencing fragments of sense.

RNA sequence* 21nt anti-sense (5′-3′)	RNA sequence[#] 21nt anti-sense (5′-3′)	Mutated nucleotide	ΔG° (anti-sense and sense)	Fragment (bp)	3′end (sense-antisense)
UAAAUGGUUGUUGUG AUUGCU	**g**AAAUGGUUGUUGU GAUUGCU	U → g	-24.2	266	3-2
	UA**u**AUGGUUGUUGU GAUUGCU	A → u	-22.1	326	2-2

*: Before cloning and sequencing, [#]: after cloning and sequencing.

After cloning and sequencing sense inserts, single-nucleotide mismatches were identified in seed. Some of these are in the region of siRNA duplexes. Mismatches were found in the region of antisense for 266 bp and 326 bp inserts (Table 4).

Construction of RNAi cassette

The antisense fragments were obtained by colony PCR via recombinant plasmids (pT-Ome1D-Whe) (266 bp+pTG19-T and 326 bp+pTG19-T) and primers. After individual digestion with *Eco*RI, *Nco*I (for 249

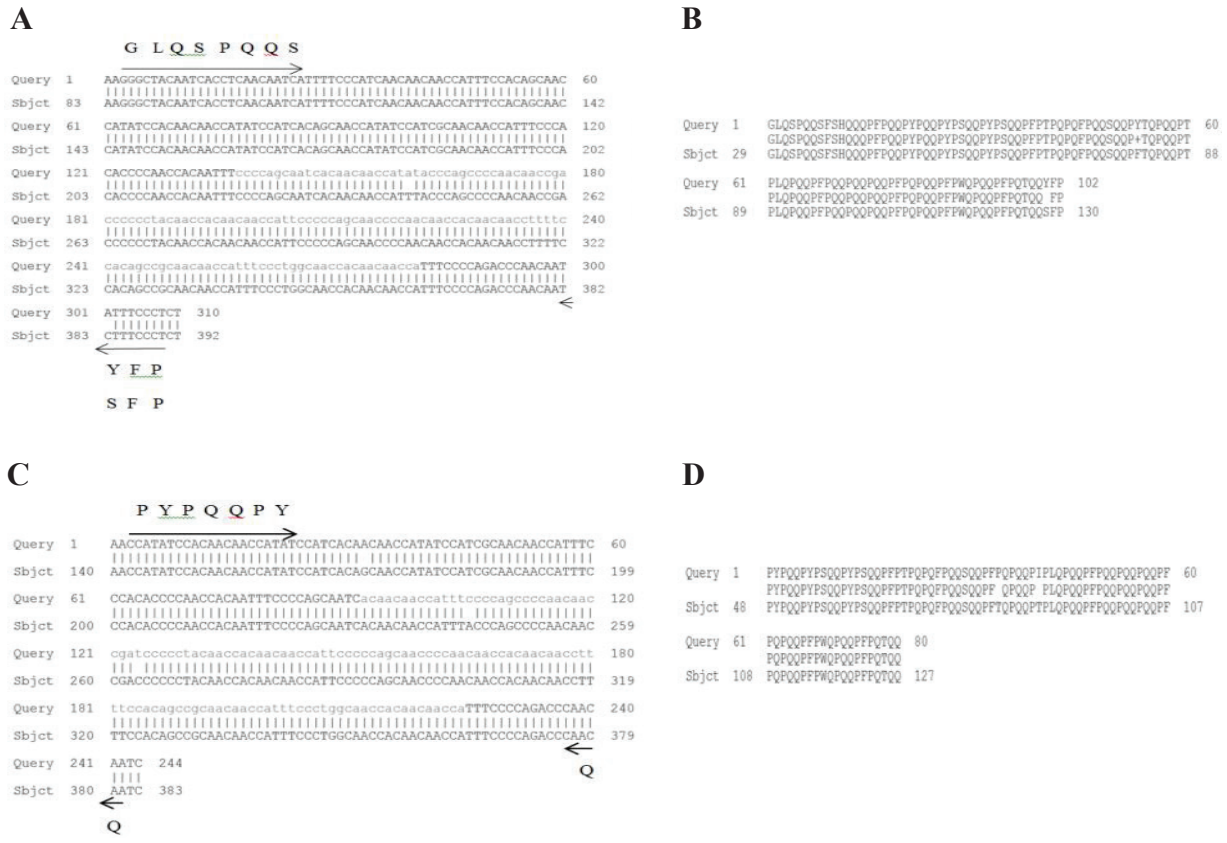

Figure 5. Comparing a sequence with sequences available in NCBI. **A:** Sequences nucleotide and **B:** protein related sense fragments of 326 bp. Other sequences are related to **C:** nucleotide sequence and **D:** protein available in sense fragment 266 bp. Sequences of protein for either fragments is contains epitopes of WDEIA and CD.

bp fragment) and *Nhe*I, *Eco*RI (191 bp fragment), two antisense fragments — omega gliadin were extracted from agarose gel. Then, the antisense fragments were ligated into pT-Ome1D-Whe (Sambrook and Russell 2001). Finally, the transfer of recombinant plasmids (sense+antisense+pTG19-T) was successfully accomplished into *E. coli* DH5α. These recombinant plasmids were confirmed by digestion with *Nco*I and *Nhe*I enzymes (Figure 6).

Ligation of hpRNAs into pAHC25 vector

Finally, hpRNAs available in the cloning vector were extracted using the gel extracting kit (Bioneer Ltd. South Korea) and ligated into pAHC25 (binary vector)(Figure 7A). This plasmid was transferred into bacterial competent cells (*E. coli* strain TOP10) and cultured in the LB medium for 24 h (Sambrook and Russell 2001). Then, the presence of two hpRNA fragments in pAHC25 were confirmed by digestion with *Sac*I enzyme (Figure 7B).

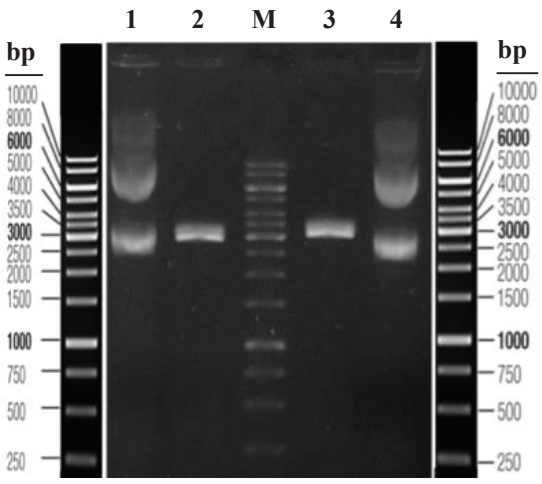

Figure 6. Final confirmation of RNAi cassette. 1 and 4. Uncut extracted recombinant plasmids 3337 bp [266 bp+191 bp+2880 bp (pTG19-T)] and 3455 bp [326 bp+249 bp+2880 bp], 2 and 3. Individual digestion with NheI, NcoI and enzymes gives band size of about 3337 bp and 3455 bp, M. 1 Kb DNA marker.

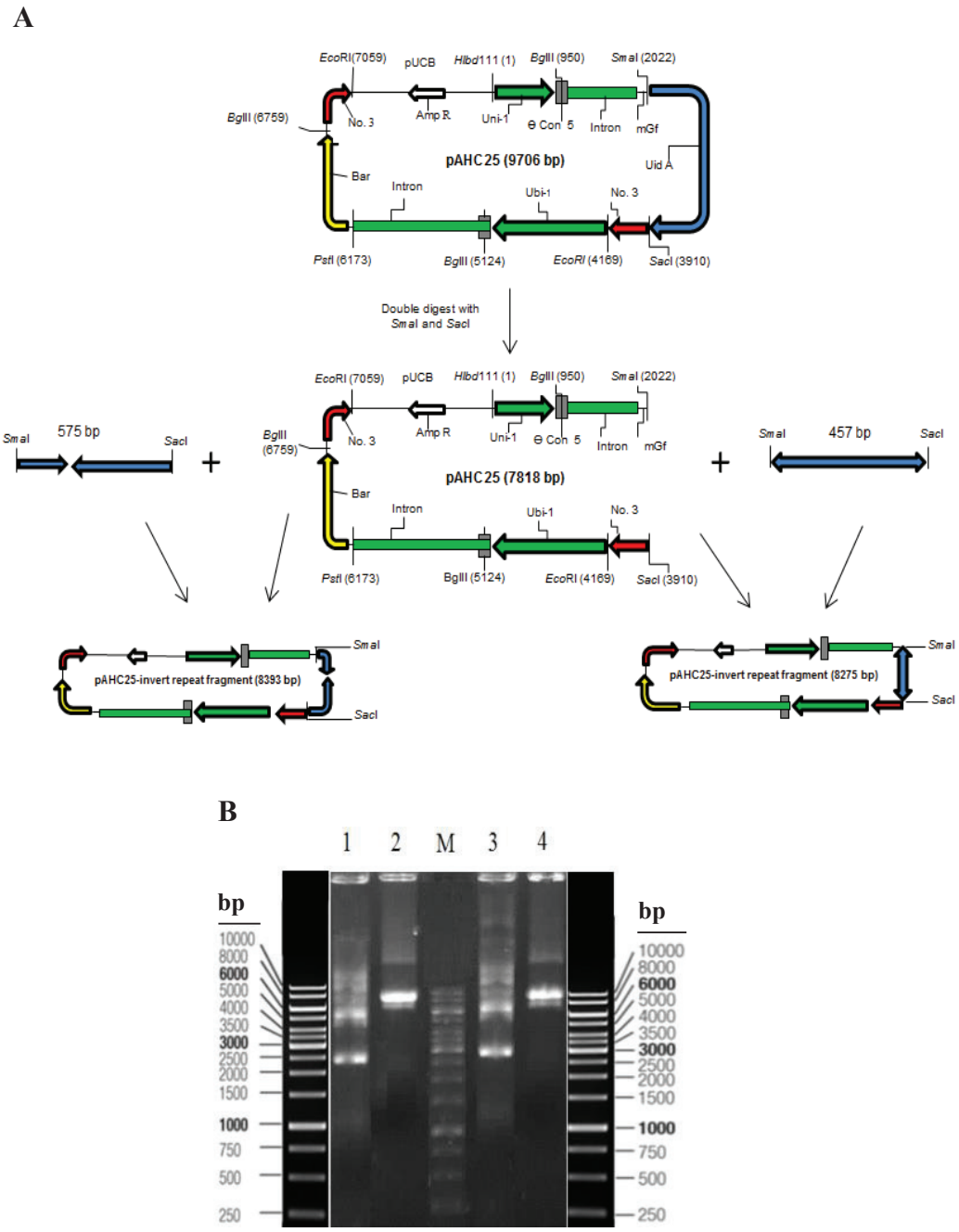

Figure 7. Showing genetic invert fragments in expression vector. **A:** Schematic diagram of RNAi construction in pAHC25 (9706 bp): Ligation of two different invert nucleotide sequences of 575 bp and 457 bp instead Uid A (1888 bp) in the binary vector, after cloning and sequencing. **B:** confirmation of cut-plasmid for either RNAi cassette was examined by *SacI* (Lanes 2-4) restriction digestion and compared with 1 kb DNA ladder (Lane M) with sizes of 8275 bp and 8393 bp. The uncut-plasmid recombinant DNAs (1-3) validated the restriction digestion.

DISCUSSION

Small interfering RNA (siRNA) is a short double stranded RNA (about 19–29 nt). It is the functional intermediate in RNAi. High efficient siRNA with 19 perfectly matched base pairs and 2 nt overhangs is one of the factors in making RNAi (Matveeva *et al.,* 2007). In the studies on siRNA (anti-sense and sense), it is specifically shown that the location of mutation (single and two-nucleotide) is of great importance (Dahlgren *et al.,* 2008). Since siRNA is highly sequence specific for gene silencing, single-nucleotide mismatches could disrupt the gene-target (on-targeting) silencing effect

(Wang and Mu, 2004). In this investigation, it was highly contemplated that several problems were raised regarding the conversion of siRNA into RNAi in target gene.

1. Effect of 5′ end start site: Dicer or RNA polymerase III prefers to initiate transcription with an A or G (purine) nucleotide. Given that, if the hairpin construct or siRNA does not start with a purine, an additional 'G' will be added to the 5′ end of the hairpin construct (Wang and Mu, 2004). In this research, about 60% and 50% of RNA sequences (anti-sense) with an initial nucleotide (at the 5′ end) in RNAi construction were used for 266 nt and 326 nt fragments, respectively.

2. In conclusion for nucleotide changes in the siRNA it was observed that the central single-nucleotide mismatches between the first 21 nt in siRNA (anti-sense) and the mRNA target are more critical than single-base mutations towards either 3′ or 5′ end. Mismatches at the 3′ or 5′ end would affect on-targeting silencing activity (Du *et al.,* 2005). Here, we analyzed combinations of single-nucleotide mismatches of the anti-sense. Therefore, after cloning and sequencing, it was shown that nucleotide alterations in the 266 nt fragment in the initial base and antisense strand (U→G) have lowered the efficiency of siRNA ability. This has occurred because it reduces identification ability of the Dicer enzyme with the free 3 nt at the 3′ end and affects free energy of the RNA (double-strand RNA of 21nt). But nucleotide alterations (A→U) were suitable for designing the 21nt siRNA.

3. Another important factor for the effect of DICER on RNAi mechanism is spacer sequence. DICER shows that efficiency is useful in spacer sequence length. By increasing the length of the sequence and creating a larger spacer, this region is easily identified by DICER (Nishimura *et al.,* 2009). Furthermore, in designing spacer it has been indicated that sequence can eventually affect gene silencing (Liu *et al.,* 2013). This procedure has been successfully practiced on *omega-2 gliadin* gene despite the fact that the repeat sequences contain higher percentage of GC than AT nucleotides. The spacer regions create 75 nt and 178 nt fragments for two 457 bp and 575 bp invert fragments, respectively.

More invert fragments of available RNAi contain two exon sequences (complementary sense and anti-sense) and one intron. For example, there is one in wheat polyglutamine (gluten) silenced for WDEIA and CD disease (Gil-Humanes *et al.,* 2010; Altenbach and Allen, 2011). In the current project, RNAi was constructed from one exon of type 1D *omega-2 gliadin* in bread wheat. This sequence includes three sections: sense, uncomplimentary (role intron) region and anti-sense, in which intron creates loop [(5′end) and (3′end)] RNAi for 266 bp and 326 bp fragments. In addition, among the advantages, the benefit of the RNAi invert fragments is the presence of two epitopes in both 266 bp and 326 bp sequences for silencing WDEIA and CD epitopes or the *omega-2 gliadin* gene. In this research, 30 gene sequences were completely used for the assessment of nucleotides by ClustalW online software. These sequences had about 80% nucleotide similarity from 1 nt to 385 nt. Given this, the application of the RNAi cassette for all 30 nucleotide sequences can completely eliminate a section of 1D chromosome containing epitopes of WDEIA and CD. In this study, a reverse fragment with high spacer sequence was ligated into pAHC25 vector. The important point concerning the recombinant plasmid is that gene silencing is highly reduced for off-targeting gene. Use of regions without nucleotide mutation after DNA sequencing is suggested to identify target epitopes associated with it.

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