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Expression of small interfering RNAs in wheat plant specific to gluten hydrolyzing proteinase (GHP) transcripts of sunnpest (*Eurygaster Integriceps*)

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Abstract
Many times alternative approaches are not available against chemical pesticides for controlling pest of economic importance in an ecofriendly manner. Sunnpest is one such pest against which till now no biological approach is available for its radical control. After coming across *Bacillus thuringensis* (*Bt*) proteins resistance in the insect pest, the latest dsRNA based siRNA approach found to be more promising for the insect control alongwith no adverse effect on the environment. The present approach involves expressing multiple synthetic siRNAs to target vital transcript or multiple transcripts of a pest. To accomplish this, siRNAs were designed by taking into consideration of avoiding the perfect off targets problem. Sunnpest (*Eurygaster integriceps*), Gluten hydrolyzing proteinase (GHP) gene has been selected as a target gene and designed multiple shRNAs sequences and cloned in a single binary vector. *Agrobacterium* based transformation was done in Wheat embryos and screened through kanamycin resistance selectivity, positive plants evaluated for shRNA expression and quantified through Real-Time PCR. The shRNA was expressed in transgenic wheat plants. Once Sunnpest feeds upon these transgenic wheat, shRNAs enters through cell sap into the insect body and converted into functional siRNAs inside the insect and specifically knock down GPH mRNA which further protects wheat seeds from GPH induced external digestion resulting in to better dough making quality wheat seeds.

Keywords: *eurygaster integriceps*, gluten hydrolyzing proteinase (GHP), small interfering RNA, real-time PCR

1. Introduction
It has been found globally that a significant amount of the economically important crop yield loss occurs due to recurring pest attacks. The use of pesticides is the only solution to prevent these attacks but regular use of chemical pesticides for long terms poses hazards to the environment. Pest controlling through ecofriendly methods is the need of hour and it could be achieved with the usage of cutting edge biotechnological techniques. Small interference (siRNA) is one of such known promising technology in this direction. siRNA is a single stranded fragment of 21-25bp sequence, is the most effective technique to be used at the post transcriptional level in the genome. These short fragments silence the expression of genes by complementarily binding and destructing specific mRNA. siRNA based expression vector approach is widely practiced in plant transformations, most of the vectors currently used in transformation contains only single siRNA expression cassette. In the present study a novel approach has been adopted which includes cloning of multiple siRNAs in a single vector with suitable promoters (targeting for single gene/ multiple genes from same organism or multiple genes from multiple organisms). To address such kind of problem a vector has been designed by harboring three shRNAs, which can produce three siRNAs and suppress the GHP gene expression (mRNA). The vector is designed in such a manner that it targets multiple sites of mRNA to achieve high efficiency in silencing Gluten Hydrolyzing Proteinase (GHP) enzymes gene expression of *Eurygaster integriceps*.

2. Materials and Methods
2.1 Sequence analysis of target Gene
Gluten Hydrolyzing Proteinase (GHP) gene of Eurygaster intergrices puton was identified as target. GHP enzyme complex is having three molecular isoforms namely GHP1 (Gene Bank ID: ADSP0606390.1), GHP2 (Gene Bank ID: ADSP0606391.1), and GHP3 (Gene Bank ID: ADSP0606392.1) [6]. These three molecular isoforms have been subjected to multiple sequence alignment analysis and identified conserved regions, which were used as targets to design siRNAs.

2.2 Construction of multiple siRNAs expression cassette for GHP gene
After predicting six siRNAs expression cassette designed, it consists of three shRNAs serially arranged with a block sequence in-between to prevent potential cross interference among the shRNAs. At 5' and 3' end of the cassettes eleven long flanking nucleotides were added. The restriction enzyme sites (REs) of EcoRI and SpeI were introduced at the two flanking sites. Three ubiquitin promoters were cloned sequentially in between EcoRI & EcoRI, SphI & BamHI and PstI & PstI respectively. Three nucleotide long insert (Ins) sequence were inserted in between two REs to keep them separate from each other. The schematic diagram of multiple shRNA expression cassettes is shown in Figure 1.

2.3. DNA sequence of GHP cassette without promoter
CCTGCTACAGTGAATTCTCAAAGCTTACATCTAGA

GCAATCGxxxCACAGAATATTCAAGAGATATTCTGT
xxxACGATTGCTTTTTTACTATGCTGTGxxxGACAGC
AGACCTCGTGCATGCTCAGGATCCGCAGGCAxxxCC
GATGTTATTCAAGAGATAACAxxxGAAGTGCCTGCT
TTTTTGATACGACAACGAAGACAGAxxxCCTCCGTC
TGCAGATGGCCAAACCTxxxGTCTTCAATTCAAGAG
ATTGAAGxxxAGAGGTTTGGTTTTTTCTGTCGTCTGG
AGCATGATACGACATCAGAACTAGTCAGCGACTTG
T

The above said sequence got chemically synthesized (ATUM Ltd. USA). DNA was isolated from the filter paper with 20-50 µl nuclease free water. Incubated the sample for 10 minutes at 37 °C, centrifuged it at 10000-12000 rpm for 5 minutes and then DNA concentration was estimated at 260/280 nm.

2.4 Transformation and retrieval of DNA

Competent cells were prepared by calcium chloride treatment method [7]. E. coli transformation was done according to heat shock method and colonies were picked by colony screening method [7]. Plasmid isolation from the selected colony was done by alkaline lysis method (Joshep Sambrook *et al*., 2001) [4].

2.5 PCR amplification of Ubiquitin from PB4NU

Ubiquitin promoter was isolated from PB4NU plasmid and transformed in to E.coli and plasmid with ubiquitin was isolated. Since, ubiquitin is having 3kb size, it is difficult for transformation. Due to this region functional region of 1kb truncated ubiquitin amplified by PCR method with specific primers. Truncated 1kb ubiquitin sequence was collected from similar work has been carried in Zea mays (DQ141598).

2.6 Cloning of shRNA cassette, Ubiquitin promoter in to pFGH vector

a. First Cloning of ubiquitin promoter obtained from PB4NU, into GHP by using PstI-PstI restriction sites

Restriction digestion with PstI RE (Thermo Scientific fast digest restriction enzymes) with 10 X Fast Digest green buffers was performed for PB4NU plasmid and GHP synthesized gene. The full length ubiquitin promoter was extracted from the gel and eluted through columns by using Qiagen gel extraction kit. The ligation of ubiquitin promoter obtained from PB4NU plasmid was done with GHP (Insert & vector ratio composition of ligation mixture was 5:1) for sixteen hours at 4°C temperature and later transformed into E.coli comp. cells by heat shock method. Colony PCR experiment was performed and confirmed by PstI digestion [Figure 4 & 5]. This was further confirmed by sequence analysis.

b. Ubiquitin promoter cloning into PGEMT vector

Ubiquitin promoter was cloned into intermediate vector PGEMT and sequences were confirmed by sequencing. First PGEMT vector was digested by HindIII & XbaI (Thermo Scientific Fast Digest restriction enzymes) with 10X Fast Digest green buffers and eluted out through columns by using Qiagen gel extraction kit. Ubiquitin promoter of PB4NU was also digested by same restriction enzyme combinations and ligated into PGEMT intermediate vector. PGEMT vector was digested by SphI and BamHI (Thermo Scientific fast digest restriction enzymes) REs with 10X FastDigest green buffers, PB4NU was also digested by same enzyme combinations and eluted ubiquitin promoter then ligated into PGEMT intermediate vector.

c. Sequential cloning of second ubiquitin promoter in GHP cassette

The GHP cassette containing first ubiquitin was digested by SphI and BamHI (Thermo Scientific Fast Digest restriction enzymes) with 10X FastDigest green buffers. Ubiquitin promoter from PGEMT intermediate vector was digested by using SphI and BamHI (Thermo Scientific fast digest restriction enzymes) REs with 10X FastDigest green buffers and eluted from gel [Figure: 6]. Ligation was performed for the above said ubiquitin promoter with GHP cassette

d. Cloning of the GHP cassette with two ubiquitin promoters into PFGC vector

The GHP cassette containing two cloned promoters (PstI-ubiquitin-PstI and SphI-ubiquitin-BamHI) digested by EcoRI and SpeI enzymes (Thermo Scientific Fast Digest restriction enzymes) with 10X FastDigest green buffers. The PFGC vector is known as transformation vector of Wheat [7]. The PFGC vector was digested by EcoRI and SpeI restriction enzymes (Thermo Scientific Fast Digest restriction enzymes) with 10X Fast Digest green buffers and eluted out from gel by using Qiagen gel extraction kit. The ligation was between PFGC and EcoRI and SpeI elutes of GHP cassette. The complete cassettes were cross checked by gel electrophoresis.

e. Cloning of third ubiquitin into PFGC vector

At this stage PFGC vector contains first and second ubiquitin promoter i.e. in between PstI & PstI, SphI & BamHI respectively. To clone third ubiquitin promoter, PFGC vector was digested with EcoRI and EcoRI restriction enzymes. The intermediate vector PGEMT, consists of ubiquitin promoter was also digested by EcoRI and EcoRI (Thermo Scientific Fast Digest restriction enzymes) with 10X FastDigest green buffers and ubiquitin fragment was eluted out by Qiagen gel extraction kit. The third ubiquitin promoter was ligated with PFGC vector.

f. Final Cloning confirmation by restriction digestion experiment

Finally vector PFGC consist first ubiquitin in between Eco RI & Eco RI, second ubiquitin in between Sp hI & Bam HI, and third ubiquitin in between PstI and PstI [Figure 7 & 8]. The restriction digestion was performed to check the all ubiquitin promoters and their correct orientations by comparing their size with 1 kb ladder (MBI Fermentas) (Joshep Sam brook *et al*., 2001) [4].

g. Transformation and evaluation

Vector pFGC5941 harboring ubiquitin promoters along with three shRNAs respectively were transferred into the Agrobactrirum transformation strain LB by using the freeze-thaw method (Gelvin 2003). Agrobacterium based transformation carried out on immature embryos of bread wheat (Triticum aestivum L.) varity Pusa Wheat – 109 (HD 2894), following method of Sivamani *et al*. 2000 [9]. Putative transgenic plants grown under kenamycin selection. Transgenic wheat lines expressing shRNAs were analysed further by using real-time PCR. Levels of shRNA1, shRNA2 and shRNA3 in transgenic wheat plants were

determined by real-time analysis using Thermo Scientific DyNAmoSYBR Green 2-Step qRT-PCR Kit and PikoRealTM Real Time system (Thermo Scientific). Total RNA was isolated from control and transgenic wheat leaves using TriZOL reagent. Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen) and primers with adapters. The amount of total RNA was normalized to β-actin gene. Reverse transcription was carried out at 60˚C for 50 minutes in order to get the cDNA and then specific primers were used to amplify the three different shRNA sequences. The real-time PCR conditions includes denaturation at 94 °C for 2 min, cycles 1-40, denaturation 94 °C for 15 sec. annealing 55 °C for 30 sec., extension 68 °C for 1 min., final extension 68 °C for 5 min., melt curve data was also recoded for each dataset. All the experiment sets were repeated thrice and each time threshold cycles (CT) were recorded. The ΔCT values were calculated and were used to find out the relative expression of the three sh RNAs using the formula $2^{-\Delta\Delta CT}$. The insect bioassay was not conducted due to non-availability of Sunnpest in India.

3. Results and discussion

In many countries, wheat serves as an important staple crop. Depending on the various conditions, it can be infested by a wide variety of insect pests. The conventional method to control insect pest is mainly dependent on the extensive use of chemical pesticides, which are harmful to the ecological system, leave considerable residual toxicity to humans and animals, and increase the production cost. Moreover, Insects have developed resistance to some of the available commercial Chemical as well as Biological pesticides. Therefore, it is desirable to develop resistant cultivars by the introduction small interfering RNA genes employing conventional genetic transformation. In this study, we used multiple sh RNAs cloning in to single vector and express in to the wheat system whole process is diagrammatically represented in figures (Fig.2 &3) three GHP 1,2&3) molecular isoforms amino acid sequences were selected as target proteins (Konaraev AV *et.al*., 2011) and downloaded from GeneBank (ADSP0606390.1., ADSP0606391.1 & ADSP0606392.1) and subjected to multiple sequence alignment through ClustalW (https://www.ebi.ac. uk/Tools/ msa/clustalw2/) program and identified the conserved regions (Fig.1) those regions were used as target regions to design sh RNAs. si RNAs are proved to interfere with target mRNAs and they will be knocked down (McManus MT et.al., 2002 [5] & Matze MA *et.al*., 2005).

Fig 1: Three GHP molecular isoforms sequences (ADP06390, ADP06391&ADP06392), were subjected to multiple sequence alignment and identified the conserved regions.

Ubiquitin is a very successful promoter in the wheat system, 3Kb size Ubiquitin sequences were isolated from PB4NU plasmid and confirmed by sequencing. 1kb truncated promoter of ubiquitin was used for the cloning experiments because complete promoter with large size may cause hindrance during cloning (Joshep Sambrook *et al*., 2001) [4]. Presence of multiple sites of HindIII, XbaI, SphI and BamHI in the PJExpress vector (which was supplied along with synthetic gene cassette by the ATUM Ltd. USA) created problem hence HindIII & XbaI direct restriction digestion could not be used for cloning (Fig.2). Hence PCR amplification was performed for cloning the second third ubiquitin promoters with above said restriction enzymes. The above said two ubiquitin promoters were initially cloned into PGEMT vector (Fig.4). However PstI site was unique in the PJExpress cassette thus cloning was performed by direct restriction digestion itself. Thus, all three sequences of ubiquitin promoter were transferred into PFGC vector (Fig 3). PFGC vector was selected because bar gene as well as Kanamycin resistance gene present in it and which is mainly used for wheat transformation (A Agrawal, et.al., 2015) [1]. Above said PFGC vector with ubiquitin promoters digested with HindIII & XbaI, SphI & BamHI and PstI & PstI REs and compared with 1kb ladder (Fig.5). To confirm the orientation of all cloned ubiquitin in the construct (Joshep Sambrook *et al*., 2001) [4].

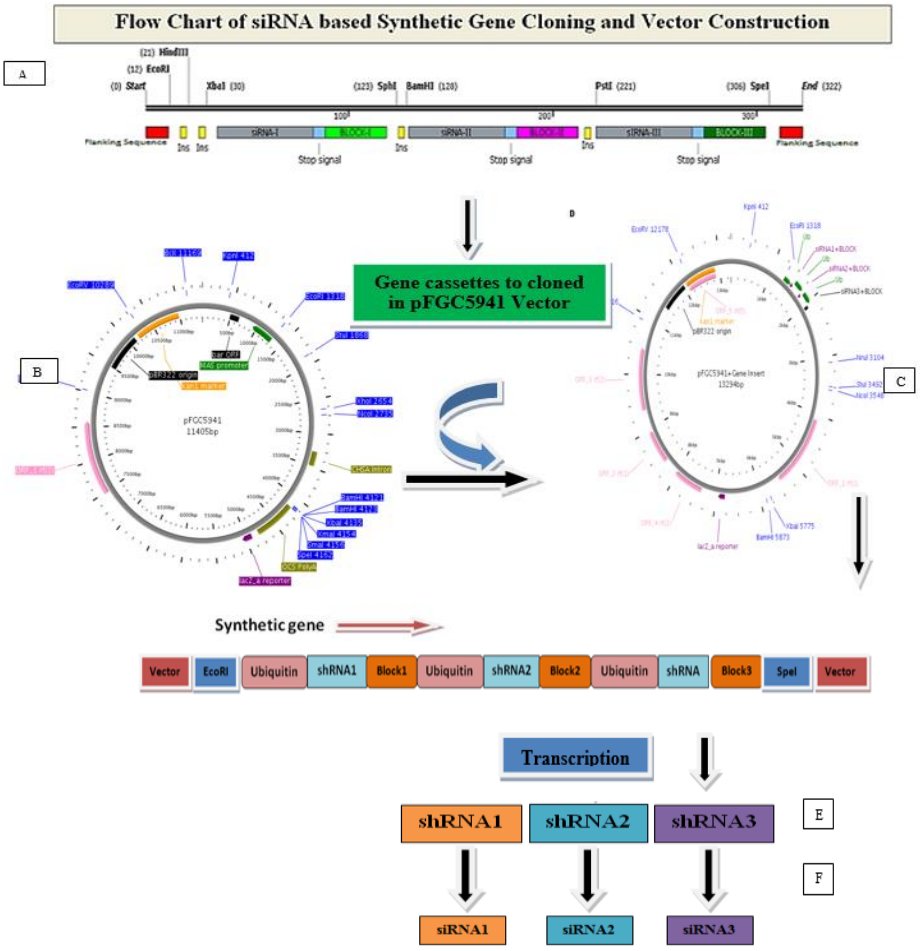

Fig 2: Flow chart showing siRNA gene development and cloning in PFGC5941 vector and their expression A. Gene cassette, B. pFGC vector, C. pFGC vector with Ubiquitin promoter, D. Synthetic Gene, E. ShRNAs & F siRNAs.

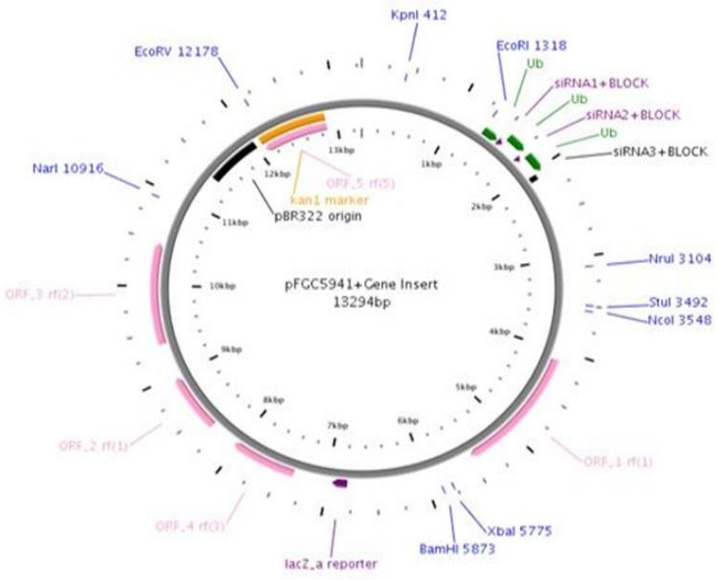

Fig 3: PFGC5941 vector and their expression with Ubiquitin promoters, shRNAs, Restriction Enzymes, Bar and Kanamycin genes.

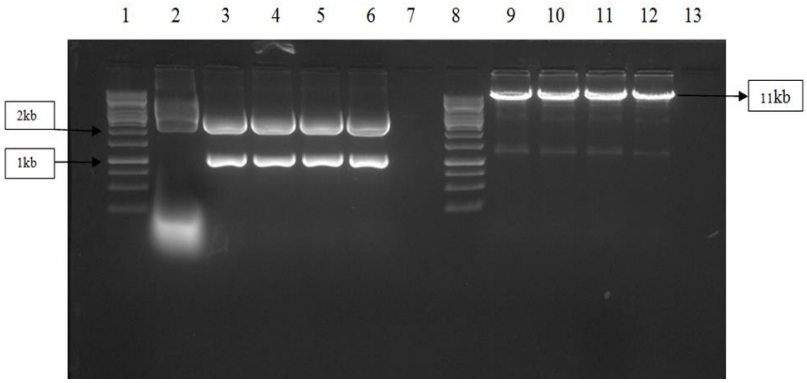

Fig 4: Digestion of PGEMT and complete cassettes of PFGC by Eco RI. column1 and 8: 1 kb ladder. column 2: control, column 3-6: PGEMT vector, column 9-12: PFGC transformation vector.

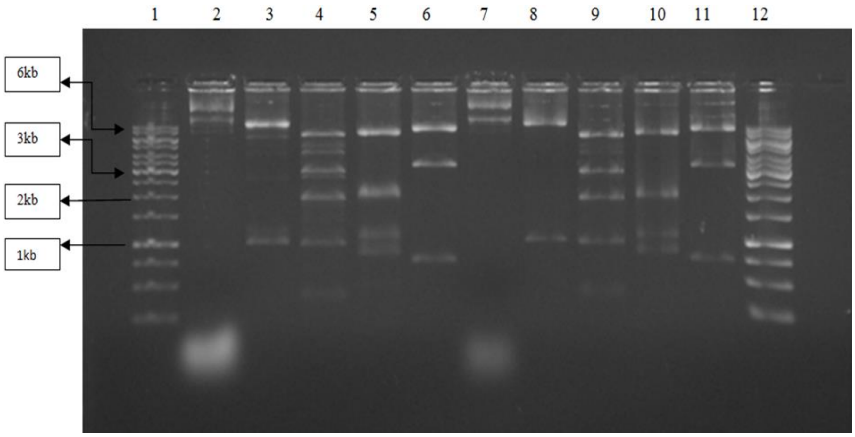

Fig 5: Above said PFGC vector with ubiquitin promoters, colum 1&12 1kb ladder, colum 2 PFGC vector, colum 3 control, colum 4 digested with HindIII & XbaI, Colum 5 digested with SphI & BamHI, colum 6 digested with PstI & PstI confirm the orientation of all cloned ubiquitin in the construct, colum 7 to 11 to check the orientation.

Wheat bread wheat (Triticum aestivum L.) variety Pusa Wheat–109 (HD 2894) precultured immature embryos collected, Agrobactirum pFGC vector alongwith cassette with best concentration (ODD_{660}=0.6), 45 minutes of inoculation and 2 days of co-cultivation and continued for shoots and rooting with different tissue culture media after 2 weeks we found shooting. Though we have bar genes but transgenic plants selection carried out through kanamycin antibiotic and shifted to soil (Cheng M, et. al., 1997 [2] & Sivamani *et al*., 2000 [9], Kamil Haliloglu & P [7]. Stephen Baenziger, 2003). Wheat leafs were selected, extracted RNA, purified and subjected to the reverse transcription and real-time PCR with suitable primers (Hala F. Eissa et.al., 2017) [3], All three shRNAs got amplified and their expression values showed that shRNA2 expressed better than two other shRNAs (Table 1& 2), relative expression also represented in graphical form (Fig 7). It shows all shRNAs successfully expressed in wheat plant.

Table 1: Real-Time PCR data showing values for shRNAs amplification with CT, CT (mean±SD), ΔCT (average) & $2^{-\Delta\Delta CT.}$

	CT (3 data set)		
25S-RNA	28.56	28.02	28.34
sh-RNA1	25.46	25.89	25.45
sh-RNA2	27.35	26.98	27.42
sh-RNA3	26.86	26.46	26.02
	CT (mean±SD)	ΔCT (average)	$2^{-\Delta\Delta CT}$
25S-RNA	24.31±0.27	0	0
sh-RNA1	25.936±0.25	-2.70667	6.788022
sh-RNA2	27.25±0.24	-1.05667	2.08724
sh-RNA3	26.45±0.42	-1.86	3.73029

Table 2: Expressing values of sh RNAs.

	Relative expression	SD
sh-RNA1	6.788022	2.167118
sh-RNA2	2.08724	0.212336
sh-RNA3	3.73029	1.104087

4. Conclusion

By using multiple si RNA approach p FGC construct developed and transformed into wheat plant, sh RNAs expression levels were evaluated and found that all three sh RNAs were expressed and will work against Sunnpest insect GHP once they enter into it through feeding on transgenic wheat.

5. Acknowledgement

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