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In-silico Analysis for Prediction of Mutational Position and Designing of sgRNA for *HDT701* Gene in Indian Rice cv. *RPBio-226*

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ABSTRACT

Background: In eukaryotes, Histone acetylation and deacetylation plays a prominent role in controlling gene expression and chromatin structure modifications. Both Histone acetyltransferases (HAT) and Histone deacetylases (HDACs) work in opposition to regulate chromatin acetylation. Reduced levels of histone H4 acetylation and increased susceptibility to the rice disease *M. oryzae* result from overexpression of HDT701 in rice. By changing the levels of histone H4 acetylation in defense-related and pattern recognition receptor (PRR) genes in rice, HDT701 reduces the activity of innate immunity which promotes resistance to *M. oryzae.* Crispr cas9 technology was created to change genes and modify characteristics, as well as to produce resistance to a variety of infections by focusing on possible biomolecules involved in plant defense mechanisms. Therefore the present study was aimed to design single guided RNA (sgRNA) and predict the gene mutational position in the HDT gene in *RPBio-226* rice cv.

Methods: To begin with, the DNA was isolated using CTAB method. Specific pair of primers was designed from the reference gene for amplification of HDT gene. In addition to, the PCR product was sequenced and the resulting sequence was applied to the creation of sgRNA. Furthermore, CHOPCHOP is a Bioinformatic search tool used to identify CRISPR–Cas single guide RNA (sgRNA) targets.

Result: The isolated genomic DNA was quantified using nanodrop and found that the concentration of the DNA was 800-1000ng/ul with the purity of 1.8. The full gene was amplified with OsHDT701 gene primers and sequenced. Based on the OsHDT701 gene sequence, oligo single guide RNA (sgRNA) was generated by using the http://chopchop.cbu.uib programme. The target site for designing sgRNA was found from 168 basepair to 190 basepair with the deletion of a nucleotide at 174th position.

Key words: CHOPCHOP, Gene mutation, *HDT701*, sgRNA, Silencing.

INTRODUCTION

Among monocots, rice is considered as a significant stable crop and also an ideal plant for functional genomics studies. For more than 50% of the world's population, it is an important part of their diet. Production and consumption of rice have dramatically expanded worldwide, rising from 450 million tonnes in 2011 to 490 million tonnes in 2020 and then projected to rise to over 650 million tonnes by 2050 (Mishra *et al*., 2018). To fulfil the rising demand for rice due to the growing population, it is estimated that output will need to expand by 40% (Vladimir *et al*., 2017). Diseases occupy a prominent position in food security worldwide and agriculture development (Kangquan *et al*., 2019). Rice blast is caused by the ascomycete filamentous fungus named *Magnaporthe oryzae* and is believed to be one of the most destructive diseases affecting rice in all rice-growing countries. Apart from the conventional plant breeding technologies, new breeding strategy has been developed with genetic engineering to enhance the rice production by improved expression or by the removal of the function of the genes which codes for the major development in the rice crop (Halim *et al*., 2021).

HDT701, a member of the plant-specific HD2 subfamily of histone deacetylases (HDACs), performs a variety of functions, in rice (*Oryza sativa* L.) and majorly involves in the innate immunity system (Bo Ding *et al*., 2012). HDT701 Transcription increases in the compatible responses whereas it decreases in the incompatible responses after the infection with the fungal pathogen Magnaporthe oryzae. Modification of *HDT701* gene in the rice cultivar is expected to obtain the plant with blast resistance. Crispr-cas9 is an encouraging gene editing tool for engineering improved agronomic traits (Fiaz *et al*., 2019) in rice through tissue

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culture and genetic transformation and to increase resistance towards biotic/abiotic stresses (Ahmad *et al*., 2020). Cas9 is a nuclease protein that led to the 20 nucleotide target DNA sequence by a single guide-RNA. The PAM sequence or the Protospacer Adjacent Motif is an 'NGG' motif found at the upstream following 20 nucleotides of the target DNA and this 20 nucleotide sequence in sgRNA work together to determine the accuracy of the target site (Zafar *et al*., 2019). The level of accuracy and specificity for knowing the target gene position depends on the sequence of sgRNA which was created based on the sequence of the target gene (reference sequence obtained from bioinformatics search). The designed sgRNA is then used for construction of the vector and then introduced into the target site of the plant cell. To edit the genes in the target site, sgRNA guides cas9 nuclease protein (Martin *et al*., 2012). Bioinformatics programmes can detect edit sites in the form of nucleotide changes to reduce off-target regions. The goal of this present study was to create single guide RNA from the sequence of rice cv. RPBio-226 DNA in order to pinpoint the position of the mutation as well as the change in the targeted region in the sequence of the gene.

MATERIALS AND METHODS

The experiment was conducted in the molecular laboratory of PPR biotech Innovations Private Limited, Hyderabad in the year 2022.

Materials include *Oryza sativa* L. cv. *RpBio-226* DNA and suitable right and left primers for PCR analysis.

DNA isolation

Isolation of the rice genomic DNA was done by taking the leaf sample and by using a standard Cetyltrimethyl Ammonium Bromide (CTAB) method (Miftahudin *et al*., 2004). Quantification of DNA was performed to measure the concentration by using a NanoDrop Spectrophotometer.

Designing of a primer and oligo sgRNA OsHDT701 using bioinformatics software

UGENE Software version 40.0 is used to design the primer sequences (Konstantin *et al*., 2012). On the basis of the reference genome, the *OsHDT701* gene sequence from *Oryza sativa* cv. Hwayoung is used as evidence. The complete database with the locus name MG715489 located on the 5th chromosome was obtained from rice NCBI. By using Bioinformatics CRISPR software tool CHOPCHOP version 3.0.0, The *OsHDT701* oligo sgRNA was constructed using the *OsHDT701* sequence from rice cv. *RpBio-226*.

PCR amplification for OsHDT701 gene

The 50 µl of PCR composition was prepared consisting of 5.0 µl genomic DNA (50 ng/µl), 5µl each of right and left primers (10 ng/µl), 10 µl of double distilled water and PCR Master mix of 25.0 µl contain Taq Polymerase enzyme, buffer, 4 mM $MgCl_2$, 0.4 mM dNTPs. The PCR programme was set on the PCR equipment with an initial denaturation temperature at 950C for 4 minutes and 30 cycles of denaturation at 95°C for 30 seconds, 55°C as an annealing temperature for 30 seconds and 72°C as an extension temperature for 30 seconds. The PCR run was subsequently terminated after 15 minutes at an extension temperature of 72°C. Out of 50 µl amplified PCR product, 10 µl of amplified PCR product was run on 1% agarose gel with 50×TAE at 100 V for 40 minutes in the electrophoreses unit. The gel was then stained with 5 µl of blue dye and is observed under BioRad-Gel Documentation Unit. The remaining 40 µl of the amplified product was then sequenced.

Data analysis

Using the ClustalW tool, the OsHDT701 gene sequence of rice cv. *RpBio-226* is matched with the referal sequence of rice cv. Hwayoung (Thompson *et al*., 1994). Search Tool for Basic Local Alignment (BLASTn) is performed in order to search rice databases for homologous nucleotide sequence.

RESULTS AND DISCUSSION

Isolation of rice genomic DNA

Isolation of rice genomic DNA from rice leaves cv. *RP Bio226* (Fig 1) was done successfully and the level of DNA purity was checked. The DNA purity ranged from 1.8 to 2.0 (260:280 nm) and DNA analysis resulted that the DNA isolated has a high purity, with an average concentration of 1377 (Table 1).

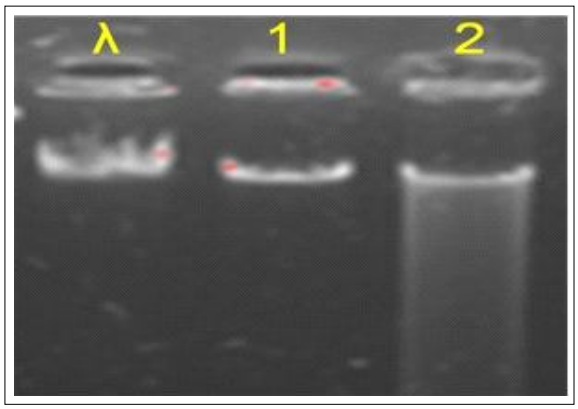

Fig 1: Electrophoresis of rice cv. *RPBio-226* DNA.
Lane λ: lambda DNA and Lane 1 and 2: *RPBio 226* sample.

Table 1: DNA quantification of rice cv. *RPBio 226*.

Sample name	Value at 230 nm	Value at 260 nm	Value at 280 nm	260/230 ratio	260/280 ratio	Concentration (ng/µl)
RPBio 226	1.042	2.070	1.090	1.986	1.899	1385
RPBio 226	1.065	2.176	1.202	2.043	1.810	1370

Gene amplification by PCR and sequence of *OsHDT701* gene from rice cv. *RPBio226*

The ORF (Open reading frame) of the *OsHDT701* gene sequence of Rice cv. *RPBio226* has been successfully used to construct both forward (left) and reverse (right) primers (Table 2). Successful amplification of the gene validated the genomic DNA cv. Hwayoung's *OsHDT701* gene sequence. An electrophoregram with a single thick band suggests that amplification was successful (Fig 2). The rice DNA amplicon size after using the *OsHDT701*F and *OsHDT701*R primers was 894 bp.

HDT701 gene sequence of rice cv. RPBio226

cgaatggagttctggggtcttgaagtcaagcctggacagactgtcaaatgtgag cctgaagatgaacgctttttgcacctttctcaggctgctcttggggaatcaaagaaagg atctgacaat gcagtaatgt atgttaaaac tgatgatcaa aagctagtcattg gaaccct ctcagctgacaagttccctc aaatccagtt tgatttggtc tttgacaaa gagtttgagctgtcacacacttcaaagactgctagtgtgttcttttctggctacaaagttt cccagccggc tgaggaagat gaaatggatt ttgattctga agaagttgaa gatgaagagg aggaagaaaa gatcattcca gctcccagggcaaatggcaa agttgaaggg aaggaaaatg agcagaaaaa acaaggcaagacagattctt cagcttcaaa atcaaaggct gcagtgaatg acgatgatga tgatgatgaca gtgatgaggatgattctgaggacgaagatctttctcctgaggatgatgatgatgattcttctg aggatgattccagcgaagatgatgaggatgagagtgacgaggaagaaactcccaagaag ccagagactggaaagaggaaagtagctgaaattgtgttgaagacaccttcgtctgat aagaaagcaaagattgctacaccgtcaggccagaagacaggtgacaagaagggtgtcc atgtagcaactccacatccggcaaagcaggctagcaagacccccgtgaatgacaagt caaagagaagtccccaaaatccggtggtgggtcaatttcttgcaagtcatgcagcaaga cgttcaacagtgaaatggctctgcaatctcactcg aaggccaagcaccccgcc aagtga.

OsHDT701 Oligo sgRNA gene

Based on the sequence of the *OsHDT701* gene sequence, the oligo single guide RNA (sgRNA) was generated using the http://chopchop.cbu.uib programme (Table 3). The knock-out action of oligo sgRNA was constructed as a negatively stranded DNA with 55 per cent of GC content, an efficiency value of 67.80 per cent with a self-complementarity of 0. The CHOPCHOP crispr was the online search tool used to determine and predict the mutational position and conformance between the sequence of oligo single guide RNA and the sequence of *OsHDT701* gene in *RPBio226* rice. The target site for designing sgRNA was found from 168 bp to 190 bp with the deletion of a nucleotide at 174th position (Fig 3).

Table 2: Primer sequence used for OsHDT701 gene amplification.

Primer	Sequence
OsHDT701F	5′-AAG CTT TAG CTC CGC CTC CCA CCT-3′
OsHDT701R	5′-ACT AGT CTT GGC GGG GTG CTT GGC-3′

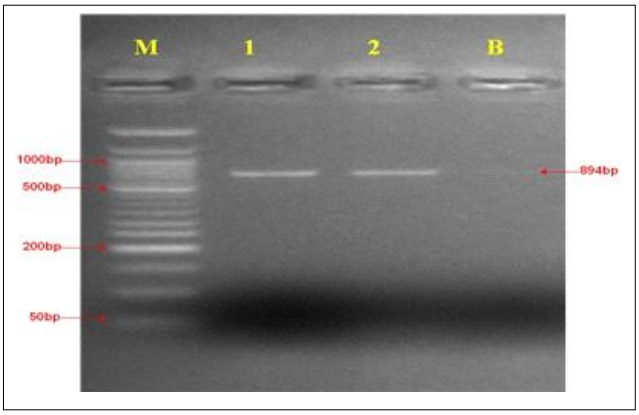

Fig 2: Electropherogram of OsHDT701 gene amplification in rice with size of 894 bp.
Lane M: 50 basepair DNA ladder; Lane 1 and 2: OsHDT701 rice gene and Lane B: Blank.

Prediction of OsHDT701 gene sequence position

BLASTn is the search engine on the NCBI website (www.ncbi.nlm.nih.gov), used to compare the gene sequence of *OsHDT701* edited rice cv. *RPBio226* to homologous database nucleotide sequences. The Expect value was found to be 0, the identity percentage was 100 and the query coverage was 100 per cent (Table 4). This result determines that the rice cv. *RPBio226 OsHDT701* gene sequence is homologous to rice cv. Hwayoung. The ClustalW tool was used to align the two *OsHDT701* gene sequences, confirming their similarity.

In our experiment, DNA isolated from our sample has high purity, which indicates no degraded DNA. Since the integrity and purity of the DNA isolated was high, genomic DNA of rice cv. RPBio226 was used as a template for amplification. Before creating sgRNA, we amplified the HDT gene sequence and double-checked its quality. sgRNA position and primer designing was done for HDT gene by utilising CHOPCHOP software version 3.0.0 (Halim *et al*., 2021). Although a list of primers can be produced, but the primer candidates were chosen depending on the position of the targeted sgRNA. Latest DNA targeting modulations, such as CRISPR activation/repression, targeted enrichment of loci for long-read sequencing and Cas9 repair outcome prediction, can also be known by this software (Kornel *et al*., 2019).

CHOPCHOP software was used to design oligo sgRNA. We used a specific action mode and a number of favourable characteristics, including GC content (per cent), low self-complementarity [0.10] and high efficiency [0%, 100%]. Hypothetically, establishment of CRISPR-Cas9 target require a

Table 3: Oligo single guide RNA sequence to target the OsHDT701 gene mutation.

Target site sequence	Genomic location on 5th chromosome	% of GC	DNA strand	Self-complementary (0,10)	Efficiency value (%)
5′GAG GGA ACT TGT CAG CTG AGA GG	29755194	55	-	0	67.80

Table 4: BLASTn result of *OsHDT701* sequence of *RPBio226*.

Details	Accession number (Acc. no)	Sample name	Acc. length	Query coverage	Expect value (E-value)	Identity percentage
Oryza sativa Hwayoung complete sequence	MG715489.1	*HDT RPBio226*	894	100%	0.0	100%

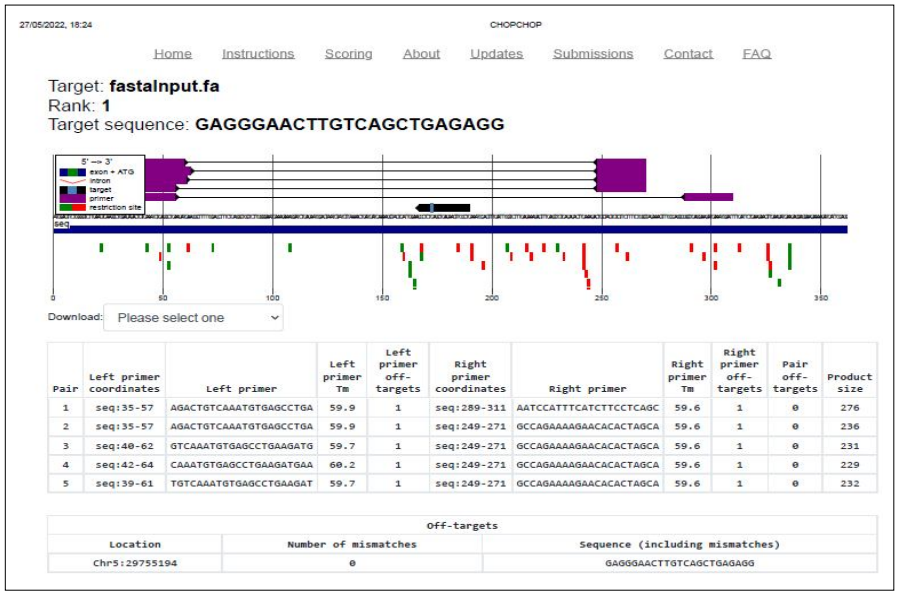

Fig 3: OsHDT gene amplification area alignment.

complementary sequence between the oligo single guide RNA and the target region. In order to increase the likelihood of gene silencing, the developed oligo sgRNA was placed nucleotide 82 from the initiation codon (Fernando *et al*., 2019). The left primer starts from 35th nucleotide to 57th nucleotide with primer Tm value of 59.9 and showing an off targets value of 1 and coming to the right primer starts from 289th nucleotide to 311th nucleotide in the sequence with the primer Tm value of 59.6 with an off targets value of 1. The analysis of the HDT gene sequence from rice cv. *RPBio226* revealed significant similarities to the HDT gene sequence from rice cv. Hwayoung.

CONCLUSION

In summary, Chop Chop was highly efficient in producing *HDT701* gene editing positions in the rice genome. Oligo single guide RNA was created successfully and it was predicted to target and suppress the HDT gene in rice cv. *RPBio226*. When *HDT701* gene is silenced in rice, histone H4 acetylation levels are increased, pattern recognition receptor and defense-related gene transcription is increased and this promotes resistance to *M. oryzae*.

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Conflict of interest

All Authors declare that they have no conflicts of interest.

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