CRISPR/Cas9 design to knockout and knockin the breast cancer gene-BRCA1 in Arabidopsis thaliana

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Abstract. Arabisopsis thaliana is a model for many molecular and genetics research especially in human research, there are many genes in A. thaliana sharing them with human. BRCA1 is a gene related with breast cancer, any damage in it sequences may leads to raise the percentage of cancer tumor. AtBCRA1 a breast cancer gene in A. thaliana plant were obtain and blast from NCBI web tool and used for gene editing, a set of PCR primers were designed to detect this gene by using in- silico tools and simulate the PCR reaction and gel electrophoresis. As well as a gene editing tool, CRISPR/Cas9 were designed to knockout and knockin any mutation in the BRCA1 gene by designing sgRNA as a target site in DNA for CRISPR action with a set of primer to amplifying the target by using CHOPCHOP web tool. A good target sequences design is a very critical step in CRISPR/Cas9 technology success.

Keywords. Arabidopsis thaliana, BRCA1, Bioinformatics, CRISPR/Cas9

1. Introduction
Gene editing is one of the most important tool to study the variation of DNA sequences which is including mutation, deletion, insertion and modification in specific site in nucleotide sequences.

The high effective tool in this field is CRISPR/Cas9 it’s including two components; a single guide RNA which is specific to target DNA (CRISPR; Clustered Regulation Interspaced Short Palindromic Repeats) and a protein (Cas9; CRISPR associated endonuclease protein 9) which is derived from Streptococcus pyogenes bacteria, [1].

It is work with single guide RNA (sgRNA) as a complex; it is containing 20 bp sequences and 3 bp as a protospacer adjacent motif (PAM) to make a double strand break (DSB) and this forced the cell to repair DSB. There are two types of repairing mechanisms: Joining of Non homologous end, the aim of this method is disrupted the gene without any protein function so the cell will be joining the DSB together by insert or delete (inDels) a number of nucleotides of not three multiplications and terminate the function of the gene by this mutation and this is called knockout, [2].

The other mechanism is Directed Repair Homology, in this method the target is to replace the gene with other sequences, so the wanted sequences are introduced in to cell with CRISPR components the cell will used the introduced sequences as a template for repairing DSB by homologous recombination and this is called knockin, [3].

2. Bioinformatics
Bioinformatics plying a very important role in success of any genetics experiments, it is depending on analyze of genome for detection any errors in nucleotides sequences and also to design PCR primers, sgRNA and other components of molecular biology researches, [4].

2.1. Arabidopsis thaliana
It is one of the most important plant in genetics and molecular biology researches, it is a small plant belong to Mustard family and contain 20k genes and consider a model of genetics studies of plants and higher organism including human. It is used as alternative tool of human for genetics researches. The Arabidopsis thaliana a member of NCBI website as a known complete genetics map beside other organism, [5].

2.2. BRCA Gene
Breast cancer gene (BRCA) is responsible of 20-45% of breast cancer of the women and there are two types of the BRCA gene; BRCA1 and BRCA2. The role of this gene is prevent cancer and repair any alteration in the DNA sequences, any mutation in the gene sequences (broken, altered) make it not work correctly and increased the possibility of cancer. The BRCA1 mutation is more frequently exists than BRCA2 and riskier [6].

3. Study Aim
The aim of this study is detecting BRCA1 gene in Arabidopsis thaliana, which is called AtBRCA1 by in-silico PCR and design CRISPR/cas9 to knockout and knockin the gene sequences.

4. Materials and Methods

4.1. Primers design
Arabidopsis thaliana BRCA1 gene (AtBRCA1) were obtain from NCBI website and then using in-silico PCR for designing specific primers to detect and amplified BRCA1 gene in Arabidopsis thaliana by using bioinformatics software tools, Geneious 11.1.5, Snapgene 4.3.11 and Amplifx 1.7.0 and check their efficiency in NCBI blast web and then simulation the PCR reaction and gel electrophoresis, [7, 8].

4.2. CRISPR/Cas9 design
In order to editing gene and for CRISPR/cas9 design; a web tool CHOPCHOP were used for the next generation and genome engineering to knock out and knock in the targeted site in the gene, it’s depend on designing a good target for a specific sit in the gene which may suffer from mutation, methylation and acetylation [9].

5. Results and Discussion

5.1. PCR primer Design
The efficient primers are a critical step to success any PCR reaction, two primers were design by using Geneious tool as shown in figure 1 and table 1.
Figure 1. Primers design by Genious tool

Table 1. Specific primers designed

| Primer         | Sequences               | Tm   | %GC | Product size | Hairpin Tm | Self-dimer | Pair dimer |
|---------------|-------------------------|------|-----|--------------|------------|------------|------------|
| Forward primer| AGCCCAATCCAGGTGACATG    | 60.0 | 55.0| 688          | NONE       | NONE       | NONE       |
| Reverse primer| GTTTCTCGGTGGGACTTCGT    | 60.0 | 55.0| 688          | NONE       | NONE       | NONE       |

In order to test if the designed primers work correctly and efficiently, they apply in NCBI website and blast them to find out the target gene, the results showed they amplify the BRCA1 gene and there are specific to it as shown in figure 2.

Figure 2. NCBI primer blast report which is showed specificity of designed primer to BRCA1
5.2. Stimulate Gel Electrophoresis

*In-silico* PCR a good tool to make all reaction virtually and to view the reaction results before apply it in lab. Snapgene tool were used to stimulate the PCR amplification of *BRCA1* gene and running the agarose gel electrophoresis, the results showed the PCR product is in size 688bp in 2% of agarose, as shown in figure 3.

![Figure 3. Simulation of 2% agarose gel electrophoresis and shown MW: Molecular weight, Line 1: BRCA1 gene amplification PCR product in size 688 bp by using Snapgene tool.](image)

5.3. Gene Editing and CRISPR/Cas9 Next Generation

Gene editing is a method to analysis the sequences of the genes and find out if there any mutation and changing take place along with DNA nucleotide arrangements. The CRISPR/Cas9 is a tool to make DNA able to rearrangement and correction any error may found. By using CHOPCHOP tool two methods were designed to editing BCRA1 gene, knock out and knock in [10].

5.4. CRISPR/Cas9 Knock out and Knock in

To knockout or knockin BRCA1 Gene by CRISPR/Cas9 the software was design many targets differ by efficiency from high to low, figure 4.
Figure 4. List of design Targets by CHOPCHOP tool to knockout or knockin BRCA1 gene in Arabidopsis thaliana

As shown in figure (4), there are many targets designed arrangement in ranks, the best target sequences is rank 1, (TGAGTGAAGATCGAAAATGGCGG) with GC 40% and efficiency 75.39 with green color in genomic location 168. An oligonucleotides were designed for amplifying the target sequences, table 2.

Table 2. Primers designed for amplifying sgRNA (Target sequences)

| Pair | Left primer | Right primer | Product size |
|------|-------------|--------------|--------------|
| 1    | TGAAAATTTTCGCCGCTTTTTATT | 60.0 | GAAATCAGGGTTTAGCTCGTC | 266 |
| 2    | TGGAAATTTTGCCTTTTATT | 60.0 | GAGACGCGATTTACAAATAGG | 227 |
| 3    | TGAAAATTTGGCCTTTTATT | 60.0 | AGCAAAGAGAGACGCGATTTAC | 235 |
| 4    | TGGAAATTTTTCGCCGCTTTTATT | 60.0 | AAATTGAGCAAGAGAGACGCG | 242 |
| 5    | TGAAAATTTGGCCTTTTATT | 60.0 | CGTCACAAAATTAGCAAGAGAG | 248 |

The best pair of oligonucleotides for amplifying sgRNA (target) is the first pair, forward or right primer GAAATCAGGGTTTAGCTCGTC while the reverse or left primer is TGAAAATTTTGCCTTTTATT.

The designing target is a very important and critical step in CRISPR method, the Cas9 protein binding in a very special site in target sequences which is called PAM, it’s contain three sequences 3’ NGG and work as a code or marker for binding Cas enzyme to the target DNA. The role of CRISPR/Cas9 is to knockout the mutation in BRCA1 gene which is a tumor inducer has located in 17q21, any changing in its position or damage increasing the possibility of cancer [11], figure 5.
Figure 5. Position of BRCA1 gene in chromatin

The CRISPR/Cas9 application on plant makes the scientists to understand and find out the results and subsequences of any gene manipulation and editing, CRISPR/Cas9 main role is to cut the double strand DNA in specific site to make the cell able to correct the error in the DNA sequences. The using CHOPCHOP web tool for designing CRISPR is good choice for planning to use this tool in a very efficient method. The CRISPR mechanisms is one out of two; repairing gene by insert or delete new sequences (indels) or by silencing the gene and prevent it to expression, figure 6. So, to make a decision of which pathway to design it may need more study of consequences of both pathways [12].

Figure 6. Mechanism work of CRISPR/Cas9
The CRISPR/Cas9 has a high advantage in genome editing in addition of it is low cost, it is easy to use in many labs which are deal with molecular biology researches. Also, the CRISPR technology doesn't need a specific protein for each gene editing, it has a general protein (Cas9) for all genes, [13]. The main role of the gene editing CRISPR is to control of epigenetic expression and modification like methylation and acetylation. The in-silico designing of single guide RNA (sgRNA) as a target for CRISPR gene editing consider a reaction key for this technology and depends on bioinformatics tools designing, [14].

References

[1] Tessa G. Montague, José M. Cruz, James A. Gagnon, George M. Church, Eivind Valen. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing, Nucleic Acids Res., 2014; 42, (1), 401–407
[2] Claude V. Campenhout, Pauline C., Anne-Clémence V., Miklos L., Agnieszka Z., Céline Sabatel, Maxime and et al. Guidelines for optimized gene knockout using CRISPR/Cas9, BioTechniques, 2019; 66(6), 295–302.
[3] Movahedi A, Almasizadehayghati A, Mohammadi K, Sangari S, Zhuge Q. Highlights of CRISPR-Cas9 Genome Editing. Methods Microbiol Mol Biol. 2018; 1(1):101
[4] Labun, K., Montague, T. G., Krause, M., Torres Cleuren, Y. N., Tjeldnes, H., and Valen, E. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. Nucleic Acids Res. 2019; 47(1),171–174.
[5] Lafarge S and Montané MH. Characterization of Arabidopsis thaliana ortholog of the human breast cancer susceptibility gene 1: AtBRCA1, strongly induced by gamma rays, Nucleic Acids Res. 2003; 31(4):1148-55.
[6] Karlapudi AP, T C V, Tammineedi J, Srirama K, Kanumuri L, Prabhakar Kodali V. In silico sgRNA tool design for CRISPR control of quorum sensing in Acinetobacter species. Genes Dis. 2018; 5(2):123-129.
[7] Tsai S.Q., Joung J.K. Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. Nat Rev Genet. 2016; 17:300–312.
[8] Naito Y., Hino K., Bono H., Ui-Tei K. CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. Bioinformatics. 2015,31:1120–1123.
[9] Lee C. CRISPR/Cas9-Based Antiviral Strategy: Current Status and the Potential Challenge. Molecules. 2019; 24(7):1349.
[10] Bernard, G.; Gaigneul, D.; Alves Dos Santos, H.; Etienne, A.; Hilbert, J.-L.; Rambaud, C. Efficient Genome Editing Using CRISPR/Cas9 Technology in Chicory. Int. J. Mol. Sci. 2019, 20, 1155.
[11] You L., Tong R, Li M., Liu Y., Xue J.and Lu Y . Mol Ther Methods Clin. Dev. 2019;13:359-370.
[12] Doench, J. G. Am I ready for CRISPR? A user's guide to genetic screens. Nat. Rev. Genet. 2018; 19, 67–80.
[13] Shan, S.;Mavrodiev, E.; Li, R.; Zhang, Z.;Hauser, B.A.; Soltis, P.S.; Soltis.D.E.: Yang, B.Application of CRISPR/Cas9 to Tragopogon (Asteraceae), an evolutionarymodel for the study of polyploidy.Mol. Ecol. Resour. 2018, 18, 1427–1443.
[14] Naim, F.; Dugdale, B.; Kleidon, J.; Brinin, A.; Shand, K.;Waterhouse, P.; Dale, J. Gene editing the phytoene desaturase alleles of Cavendish banana using CRISPR/Cas9. Transgenic Res. 2018, 27, 451–460.