NAHEP sponsored

Short term training program on

Genome editing of crops: Methods and Applications

December 27, 2019 to January 8, 2020

Course Director

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About NAHEP-CAAST at ICAR-IARI, New Delhi

Centre for Advanced Agricultural Science and Technology (CAAST) is a new initiative and student centric subcomponent of World Bank sponsored **National Agricultural Higher Education Project (NAHEP)** granted to The Indian Council of Agricultural Research, New Delhi to provide a platform for strengthening educational and research activities of post graduate and doctoral students. The ICAR-Indian Agricultural Research Institute, New Delhi was selected by the NAHEP-CAAST programme. NAHEP sanctioned Rs 19.99 crores for the project on "**Genomic assisted crop improvement and management**" under CAAST programme. The project at IARI specifically aims at inculcating genomics education and skills among the students and enhancing the expertise of the faculty of IARI in the area of genomics.

Objectives:

1. **To develop online teaching facility and online courses for enhancing the teaching and learning efficiency, and scientific communications skills**
2. **To develop and/or strengthen state-of-the art next-generation genomics and phenomics facilities for producing quality PG and Ph.D. students**
3. **To develop collaborative research programmes with institutes of international repute and industries in the area of genomics and phenomics**
4. **To enhance the skills of faculty and PG students of IARI and NARES**
5. **To generate and analyze big data in genomics and phenomics of crops, microbes and pests for genomics augmentation of crop improvement and management**

IARI's CAAST project is unique as it aimed at providing funding and training support to the M.Sc. and Ph.D. students from different disciplines who are working in the area of genomics. It will organize lectures and training programmes, and send IARI students and covering students from several disciplines. It will provide opportunities to the students and faculty to gain international exposure. Further, the project envisages developing a modern lab named as **Discovery Centre** that will serve as a common facility for students' research at IARI.

Core-Team Members:

S.No.	Name of the Faculty	Discipline	Institute
1.	Dr. Ashok K. Singh	Genetics	ICAR-IARI
2.	Dr. Vinod	Genetics	ICAR-IARI
3.	Dr. Gopala Krishnan S	Genetics	ICAR-IARI
4.	Dr. A. Kumar	Plant Pathology	ICAR-IARI
5.	Dr. T.K. Behera	Vegetable Science	ICAR-IARI
6.	Dr. R.N. Sahoo	Agricultural Physics	ICAR-IARI
7.	Dr. Alka Singh	Agricultural Economics	ICAR-IARI
8.	Dr. A.R. Rao	Bioinformatics	ICAR-IASRI
9.	Dr. Ramcharan Bhattacharya	Molecular Biology & Biotechnology	ICAR-NIPB
10.	Dr. K. Annapurna	Microbiology (Nodal officer, Grievance Redressal, CAAST)	ICAR-IARI
11.	Dr. R. Roy Burman	Agricultural Extension (Nodal officer, Equity Action Plan, CAAST)	ICAR-IARI
12.	Dr. K.M. Manjaiah	Soil Science & Agri. Chemistry **(Nodal officer, CAAST)**	ICAR-IARI
13.	Dr. Viswanathan Chinnusamy	Plant Physiology **PI, CAAST**	ICAR-IARI

Associate Team

S.No.	Name of the Faculty	Discipline	Institute
1.	Dr. Kumar Durgesh	Genetics	ICAR-IARI
2.	Dr. Ranjith K. Ellur	Genetics	ICAR-IARI
3.	Dr. N. Saini	Genetics	ICAR-IARI
4.	Dr. D. Vijay	Seed Science & Technology	ICAR-IARI
5.	Dr. Kishor Gaikwad	Molecular Biology & Biotechnology	ICAR-NIPB
6.	Dr. Mahesh Rao	Genetics	ICAR-NIPB
7.	Dr. Veena Gupta	Economic Botany	ICAR-NBPGR
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11.	Dr. Lekshmy S	Plant Physiology	ICAR-IARI
12.	Dr. Madan Pal	Plant Physiology	ICAR-IARI
13.	Dr. Shelly Praveen	Biochemistry	ICAR-IARI
14.	Dr. Suresh Kumar	Biochemistry	ICAR-IARI
15.	Dr. Ranjeet R. Kumar	Biochemistry	ICAR-IARI
16.	Dr. S.K. Singh	Fruits & Horticultural Technology	ICAR-IARI
17.	Dr. Manish Srivastava	Fruits & Horticultural Technology	ICAR-IARI
18.	Dr. Amit Kumar Goswami	Fruits & Horticulture Technology	ICAR-IARI
19.	Dr. Srawan Singh	Vegetable Science	ICAR-IARI
20.	Dr. Gograj S Jat	Vegetable Science	ICAR-IARI
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22.	Dr. V.K. Baranwal	Plant Pathology	ICAR-IARI
23.	Dr. (Ms.) Deeba Kamil	Plant Pathology	ICAR-IARI
24.	Dr. Vaibhav K. Singh	Plant Phathogy	ICAR-IARI
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29.	Dr. V. Govindasamy	Microbiology	ICAR-IARI
30.	Dr. S.P. Datta	Soil Science & Agricultural Chemistry	ICAR-IARI
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32.	Dr Satyapriya	Agricultural Extension	ICAR-IARI
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34.	Dr. Seema Jaggi	Agricultural Statistics	ICAR-IASRI
35.	Dr. Anindita Datta	Agricultural Statistics	ICAR-IASRI
36.	Dr. Soumen Pal	Computer Application	ICAR-IASRI
37.	Dr. Sanjeev Kumar	Bioinformatics	ICAR-IASRI
38.	Dr. S.K. Jha	Food Science & Post Harvest Technology	ICAR-IARI
39.	Dr. Shiv Dhar Mishra	Agronomy	ICAR-IARI
40.	Dr. D.K. Singh	Agricultural Engineering	ICAR-IARI
41.	Dr. S. Naresh Kumar	Environmental Sciences; **Nodal officer, Environmental Management Framework**	ICAR-IARI

Foreword

The ICAR-Indian Agricultural Research Institute (IARI), New Delhi is the seat of green revolution in India, and continues to contribute to the food and livelihood security of the Nation. Besides, it enormous research contributions, IARI is the premier Institute for higher education in Agriculture in the Country. ICAR-IARI greatly propels both research and academics in hand in hand. The ICAR-National Institute for Plant Biotechnology, New Delhi has made striding contributions in the field of agricultural research for crop improvement in India. The institute has core strength in the field of genomics and transgenic development for conducting advanced research in plant biotechnology. International and national genome sequencing projects on rice, wheat, tomato, pigeonpea, mango, *Mesorhizobium, Puccinia and Magnaporthe* have been successfully executed. I am happy to learn that under **National Agricultural Higher Education Project (NAHEP) on Centre for Advanced Agricultural Science and Technology (CAAST)**, **ICAR-IARI** in collaboration with **ICAR-NIPB**, New Delhi will be organizing a training Programme on ***"Genome Editing of Crops: Methods and Applications"*** from 27th December 2019 to 8th January 2020 to import skills to the PG students in this emerging area.

Targeted genome editing emerged as prevalent technique as next generation breeding tool for crop improvement. CRISPR/Cas system was developed in this regard for the crops like rice and soybean at IARI. The mutants developed through this platform depict promising plant phenotypes and biochemical characteristics. At the Institute, genome editing research is in progress for enhancing yield and stress tolerance in rice and quality improvement in soybean. With expertise available in NIPB and IARI Faculty, I am sure that that this training will provide a great exposure to the post-graduate students in the field of molecular biology and genome editing.

(Rashmi Aggarwal)
Dean & Joint Director (Education)

Preface

Food production must be doubled by 2050 to meet the demands of burgeoning population. In addition to the food, Agriculture must also cater to the needs of feed & fodder, fibre and fuel requirements of human kind. These demands must be met under the scenario of diminishing natural resources and global climate change. Conventional methods of development of crop varieties takes longer time and often limited to already available traits within the crossable gene pool.

Genome editing serves as promising tool to meet out the demands by aiding rapid and precise improvement of crops. CRISPR/Cas system and its latest versions are exhibiting its potentiality on targeted editing by insertion, deletion or frame shift of nucleotides of the genes of interest for accelerating plant growth and development. CRISPR/Cas system is the most flamboyant system of third generation of sequence specific nucleases for enabling double strand breaks at any desired site of any target in the genome which is subsequently healed by DNA repairing mechanisms like NHEJ (Non homologous end joining) and HDR (Homology directed recovery). Several improvements in nuclease versions such as Cpf1, Cas13 and in base editing, multiplexing, advanced delivery of the gene constructs, proper vector and target selection, gRNA design to avoid off targets and efficient construction of gRNA-Cas9 cassette were continuously rendered in order to improve its functionality and applicability in the plant cells. Since, it is precise, faster and easy technique to construct CRISPR have widespread applications in rice, wheat, maize, sorghum, barley, tomato, sweet orange, apple, cotton, lettuce, soybean, citrus, lotus, petunia, mushroom, and other crops. Eventually, genome edited crops shows appreciable differences in plant growth, yield, quality, biotic and abiotic stress resistance.

The greater goal of this training is to impart knowledge and training to the post graduate students on CRISPR/Cas systems with special emphasis on its molecular biology. Certainly, students could be equipped with technical training starts from gRNA design to CRISPR gene construction, development of genome edited lines and identification of genome edited plants in greater details. The theory classes along with practical schedules eventually lend to the networking opportunities with the scientists and experts through scientific discussion that acuminates students' skills in the area of genome editing of plants.

Dr. Viswanathan Chinnusamy
Dr. Ramcharan Bhattacharya
Dr. Anirban Roy
Dr. Archana Watts
Dr, Anshul Watts
Dr. Shivani Nagar
Dr. Nagaraj Kumar M

Acknowledgments

1. Secretary DARE and Director General ICAR, New Delhi
2. Deputy Director General (Education), ICAR, New Delhi
3. Assistant Director General (HRD), ICAR, New Delhi
4. National Coordinator, NAHEP, ICAR, New Delhi
5. CAAST Team, ICAR-IARI, New Delhi
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7. Director, ICAR-IARI, New Delhi
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9. Dean & Joint Director (Education), ICAR-IARI, New Delhi
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11. Head, Division of Plant Pathology, ICAR-IARI, New Delhi
12. Professor, Division of Plant Pathology, ICAR-IARI, New Delhi
13. AKMU, ICAR-IARI, New Delhi
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15. Staff & students, ICAR-NIPB, New Delhi
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NAHEP-CAAST sponsored training programme on
"Genome Editing of Crops: Methods and Applications"
from December 27, 2019 to January 8, 2020

Organized by

ICAR-National Institute for Plant Biotechnology, New Delhi &
ICAR-Indian Agricultural Research Institute, New Delhi

Schedule of training program

Date	Time	Lecture/Practical	Faculty
27-12-2019	10:30-11:30h	Inauguration	GS Sirohi Conference Hall, Division of Plant Physiology, IARI, New Delhi
	12:00-12:30h	Pre-training Evaluation	Dr. Anirban Roy & Co-Coordinators
	12:30-13:30h	Land mark events and heroes of CRISPR	Dr. Anirban Roy Division of Plant Pathology, IARI, New Delhi
	14:30-17:30h **Practical**	GuideRNA design-Tools	Dr. Anshul Watts & Dr. R.C. Bhattacharya NIPB, New Delhi
28-12-2019	10:00-11:00h	Genomic Resources and gate ways of their utilization	Prof. N. K. Singh Director, NIPB, New Delhi
	11:30-12:30h	Mechanism of genome editing (CRISPR	Dr. R.C. Bhattacharya NIPB, New Delhi
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	*In vitro* validation of gRNA	Dr. Anirban Roy Division of Plant Pathology IARI, New Delhi & Dr. Anshul Watts & Mr. Ritesh Kumar Raipuria, NIPB, New Delhi
29-12-2019		Sunday	
30-12-2019	10:00-11:00h	Knock down vs knock out of genes in trait modification	Dr Sunil Mukherjee Division of Plant Pathology, IARI, New Delhi
	11:30-12:30h	Recent developments in CRISPR variants	Dr. Deepak Bisht NIPB, New Delhi
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	Construction of genome editing vector – Ligation, transformation & Multiplexing sgRNA constructs	Dr. R.C. Bhattacharya NIPB, New Delhi & Dr Anirban Roy Division of Plant Pathology IARI, New Delhi &

			Dr. Anshul Watts & Mr. Ritesh Kumar Raipuria, NIPB, New Delhi
31-12-2019	10:00-11:00h	Potential application of CRISPR in improving plant traits	Dr. M.K. Reddy, Group leader, Crop Improvement ICGEB, Aruna Asaf Ali Marg, New Delhi-110 067
	11:30-12:30h	CRISPR-Cas technology as next generation breeding tool	Dr. Ashok K Singh Joint-Director Research IARI, New Delhi
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	Construction of genome editing vector –plasmid isolation and confirmation by PCR	Dr. R.C. Bhattacharya & Dr. Anshul Watts & Mr. Ritesh Kumar Raipuria, NIPB, New Delhi
1-1-2020	10:00-11:00h	Improving fungal disease resistance in plants through Genome editing	Dr. Aundy Kumar Division of Plant Phathology, IARI, New Delhi
	11:30-12:30h	Genome editing in virus management in crops	Dr Anirban Roy Division of Plant Pathology, IARI, New Delhi
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	*In vivo* validation – Transient by Agroinoculation & validation by T7 nuclease assay	Dr. Anirban Roy Division of Plant Pathology, IARI, New Delhi
2-1-2020	10:00-11:00h	Genome editing in Insect/Nematodes	Dr. Subramanian S Division of Entomology, IARI, New Delhi
	11:30-12:30h	Genome editing for human health management	Dr. Sivaprakash Ramalingam Principal Investigator, Genome Engineering and Stem Cell Laboratory, CSIR-IGIB, Sukhdev Vihar, New Delhi - 110 025
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	*In vivo* validation – Protoplast transformation & Agrobacterium mediated transformation	Dr. N.C. Gupta & Dr R.C. Bhattacharya NIPB, New Delhi
3-1-2020	10:00-11:00h	Genome editing in crops for nutritional quality	Dr. Siddharth Tiwari National Agri-Food Biotechnology Institute (NABI), Main Campus, Sector 81, Sahibzada Ajit Singh Nagar, Punjab – 140306

	11:30-12:30h	Genome editing in Animals	Dr. Sachinandan De Principal Animal Genomics Lab. Animal Biotechnology Centre. National Dairy Research Institute Karnal-132001, Haryana
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	DNA & RNA isolation from CRISPR-Cas9 lines	Ms. Pragya Yadav, Mr. Shashank K. Yadav & Dr. Archana Watts Division of Plant Physiology, IARI, New Delhi
4-1-2020	10:00-11:00h	Genome editing in improving abiotic stress tolerance in crops	Dr. C. Viswanathan Division of Plant Physiology, IARI, New Delhi
	11:30-12:30h	Visit to NPF & Phenomics facility	
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	Identification of CRISPR-Cas edited mutant plants by Heteroduplex assay & RE analysis	Dr. Monica Saifi & Dr. Shivani Nagar Division of Plant Physiology, IARI, New Delhi
5-1-2020		Sunday	
6-1-2020	10:00-11:00h	Regulation of GE products in India	Dr. S.R. Bhat Emeritus Scientist NIPB, New Delhi - 110012
	11:30-12:30h	Regulation of genome edited crops- Global Scenario	Dr. Amitabh Mohanty National Genomics and Genotyping Facility (NGGF)-DBT NIPGR, New Delhi-110067
	12:00-13:00h	Progress Review & Discussion	Course Director/Coordinators
	14:30-17:30h **Practical**	Identification of CRISPR-Cas edited mutant plants by PCR-Fragment length polymorphism & DNA sequence analysis	Mr. Santosh Kumar VV & Dr. Nagaraj Kumar M. Division of Plant Physiology, IARI, New Delhi
7-1-2020	10:00-13:00h	Group discussion on students' research proposal	Course Director, Coordinators, Co-coordinators, CAAST-Core Team
	14:30-17:30h **Practical**	Real time-qPCR for detection of virus in genome-edited lines	Dr. Anirban Roy Division of Plant Pathology, IARI, New Delhi
8-1-2020	10:00-10:30h	Post-training evaluation	
	11:00-12:30h	Valedictory Function	

Contents

Lecture Notes

CRISPR/Cas genome editing for abiotic stress tolerance of crops

M. Nagaraj Kumar*[1] and Viswanathan Chinnusamy[1]

**Ramalingaswami Fellow-DBT*

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Abundance of pollutants and their distribution in the environment mainly cause the fluctuations in the temperature and rainfall distribution which altogether lead to severe episodes of drought, heat and cold stresses. Indeed, abiotic stress is one of the major limiting factors affecting plant growth and yield. It was reported that increase in 1^0 C causes significant yield losses in rice, wheat and maize (Yang *et al.*, 2017; Wang *et al.*, 2019). However, agricultural production has to be increased more than fifty percent before 2050 to meet out the food security. To tackle this challenge, adoption of advanced molecular breeding techniques has to be deployed.

Despite burgeoning publications are being out in the field of stress biology, the wholesome solution to the problems of drought, salt and cold stresses still remains fragmentary. This could be mainly because of; stress tolerance is a complex trait regulated by multitude of signaling pathways corresponded with number of gene regulators. So, in order to combat with multitude of stressors with complex regulators, a powerful weapon is the need of the hour. CRISPR/Cas system is one such prevalent and dominant genome editing tool through which crop improvement have being done extensively through targeted modification of the genetic loci/locus of interest. After the advent of CRISPR/Cas system, the development of efficient crops to withstand against adverse stressors like drought, salt and cold and its combinatorial attack is getting closer to the reality. CRISPR/Cas9 system comprises of single effector Cas endonuclease and chimeric guide RNAs (SgRNA) which together forms Ribonucleic Protein complex (RNP) for introducing double strand breaks at the protospacer adjacent motif (PAM) sequences of the desired target. Subsequently, by the cell's own repairing machinery, breaks at nucleotides are fixed thereby resulting 'indels' in the gene of interest.

ABA is a well-known stress hormone controls stress associated signaling pathways in response to different kinds of stress. It is perceived by PYL receptors which are 13 genes in rice. Knocking out those 13 genes in rice by CRISPR/Cas9 resulted the increased rice productivity as well as plant growth. Among the mutated lines, group I (*PYL1* to *PYL6* and *PYL12*) showed more plant growth while maintaining other traits (Miao *et al.*, 2018). Similarly, *TaDREB2* and

TaDREB3 in wheat protoplast were edited by CRISPR with almost of 70% efficiency, demonstrated the increased drought tolerance than wild type (Kim *et al.*, 2018). *SAPK2*, one of the MAPK family member in rice and *SIMAPK3* as like in Tomato were mutated by CRISPR/Cas9 and showed the increased tolerance to drought and salt stress as well (Lou *et al.*, 2017, Wang *et al.*, 2017a). Beyond editing, CRISPR system could be used for activation or repression of the targets by using catalytically inactive form of Cas9 called dCas9. Recently, it was reported that dCas9 fused with chromatin modulator, HAT (Histone acetyl transferase) was used for activating the expression of AREB2 (ABA responsive element binding protein) which regulates several downstream genes of ABA signaling in response to drought stress (Roca Paixao *et al.*, 2019).

CBF genes are imparting tolerance to cold stress in plants however it is multi member gene family, for example 12 CBFs in case of rice and 3 in Arabidopsis. CRISPR/Cas9 was deployed to generate *cbf1cbf2* double mutant and *cbf1cpf2cpf3* triple mutant to study the significance of individual CBFs in cold stress tolerance (Jia *et al.*, 2016). Regulation of stomatal density and stomatal index is an important trait for water use efficiency. CRISPR/Cpf1 was used to edit one of the regulator of stomatal density, *OsEPFL1* which resulted improved stomatal patterning in rice under stress conditions (Yin *et al.*, 2019). *SlNPR1* is the ortholog of Arabidopsis *NPR1* which regulates both abiotic and biotic resistance. The role of *SlNPR1* in drought was found by editing through CRISPR and found that *slnpr1* mutant shows increased sensitivity to drought coupled with higher stomatal aperture and electrolyte leakage (Li *et al.*, 2019b). Despite the above evidence show the potential of gene editing in generating stress tolerance in crop plants, still many stress-associated potential genes have to be edited by CRISPR for ever-resistant crops against stressors. Even though, several evidences are keep coming up for CRISPR edited plants, still many abiotic stress resistance genes have to be targeted for genome editing. Structural genes, regulatory genes and cis- regulatory sequences for drought, heat and cold stresses are extensively reported. So, potential genes from those reports could be exploited for CRISPR based approaches for tackling abiotic stress. In figure 1, few examples of genes edited by CRISPR/Cas system for abiotic stress resistance was depicted.

The potential of genome editing in crops is getting enormous and growing tremendously. However, the discrepancies exist for the GE crops in terms of its safety and adaptation. The assessment of CRISPR crops for its direct or long-term effect as a food and as feed, varies from

countries to countries for its acceptance. It affects the trading of GE crops between two countries with different legislative procedures. So, the co-ordination in the legal policy should be standardized at the global scale to increase the marketability of CRISPR/Cas crops in future.

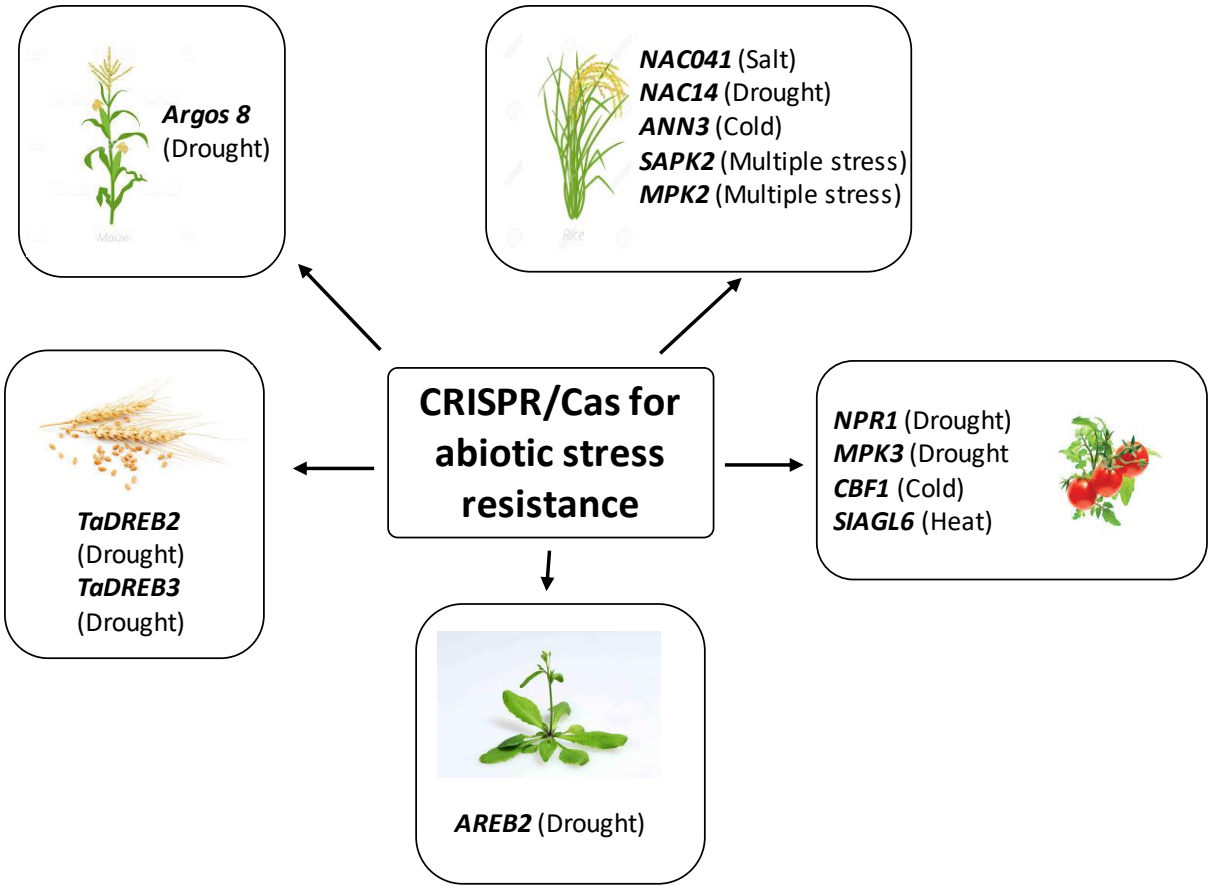

Figure 1. Some of the examples of CRISPR/Cas edited crops showing resistance to abiotic stressors

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Editing rice-genome with CRISPR/Cas9: To improve agronomic traits for increased productivity

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Increasing yield is one of the basic objectives of rice crop improvement programs. The success of conventional breeding to improve rice productivity relies on the availability of beneficial genetic variation in the population for introgression of the selected traits into an elite background. However, pyramiding widely dispersed beneficial allele from wide spectrum of genetic backgrounds i.e. landraces/wild relatives into commercially cultivated rice cultivars is very time consuming and also introduces several deleterious alleles along with due to linkage drag in the conventional breeding processes. In addition, the availability of such beneficial alleles to rice breeding community becomes a bottleneck. Alternatively, the introduction of foreign DNA into plant genomes through transgenic approach has been a focus of crop improvement. However, the transgenic approach does not harness a plant's native genetic repertoire to create traits of agricultural value. The public concerns over the incorporation of transgenes from distantly related organisms into crop plants and the governments regulatory safety concerns have impeded the widespread use of transgenic approach for crop improvement.

With the recent discovery of targeted genome editing technology it becomes easy to introduce the required genetic variability into any established rice cultivar directly by targeted genomic sequence alterations without any requirement of actual rice mutants and the time-consuming plant breeding processes. Even though the genome editing tools were initially introduced into the plant by transgenic approach, it is often unlinked to the site of DNA modification and the genome editing tools can be removed later by segregation in subsequent generations, leaving a non-transgenic plants that carries only the desired edited DNA sequence to create traits of agricultural value in crop plants.

Currently, targeted genome editing is carried out using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced

short palindromic repeats (CRISPR)/Cas9. Both ZFNs and TALENs were artificial fusion proteins comprising an engineered DNA-binding domain fused to the nonspecific FokI nuclease domain. Both ZFNs and TALENs function as dimers; one of the monomer partners recognizes a DNA sequence on one strand and the other partner recognizes another DNA sequence nearby on the opposite strand. The spacing of DNA binding distance between the two monomers of either ZFNs and TALENs is very critical to allow the FokI nuclease domains to dimerize and cause a double strand DNA breaks. ZFNs and TALENs can theoretically target to any region of the genome by engineering the DNA binding domains as per the target nucleotide sequence information. However, to engineer these sequence-specific nucleases with the requisite DNA specificity for each target site in the genome is a bottleneck, very difficult, laborious and expensive. Some of the ZFNs and TALENs pairs fail to generate the anticipated double stranded DNA breaks in vivo because of methylation of target sequences.

The Cas9 is a naturally encoded monomeric polypeptide with two separate DNA endonuclease domains, which is the most recent addition to the targeted genome editing toolbox. CRISPR/Cas9 is the part of adaptive immune system of bacteria, protecting them against invading nucleic acids such as plasmids and viruses by cleaving the foreign DNA in a sequence-dependent manner. The transition of naturally encoded bacterial Cas9 DNA endonuclease from bacterial adaptive immunity to become a genome-engineering tool when expressed in any heterologous system including plants. The Cas9 introduces double strand DNA breaks in any defined region of the genome guided by 20 base guide-RNA sequence. Unlike ZFNs and TALENs, Cas9 can edit several target genomic regions simultaneously as multiplexing without bothering the methylation status of target region.

The double-strand- DNA breaks were generated in the genome where the DNA editing was desired using Cas9. These DNA breaks were frequently repaired by non-homologous end-joining (NHEJ) results in deletion or addition of few nucleotides causes a frame-shift and/or gene knockout. Sometimes the double stranded DNA breaks were repaired by homologous recombination to introduce the desired sequence variation and/or targeted gene knock-in through appropriate repair template along with regions of homology to the sequence surrounding the double strand break. However, the precise selective nucleotide substitution using homologous recombination-based genome editing is more challenging to implement because the cleavage of DNA by Cas9 must be coordinated with delivery of the DNA repair

template and subsequent selection and/or identification of properly edited cell line/plant. In addition, the modified Cas9 system was used for regulation of gene expression, base editing and epigenetic modifications etc. For the recent past several plant genomes were edited using Cas9 suggesting its broad applicability by various transformation platforms, i.e. protoplast transfection, agro-infiltration and generation of stable transgenic plants.

NHEJ is the most common double strand break repair mechanism in most organisms, including higher plants. The genome editing for targeted regions through NHEJ repair often generate gene knockouts. Gene knockouts are valuable for identifying gene function but have limited utility in terms of trait development. However, the decades of research efforts of rice research-community identified several rice loci that negatively regulate agronomic performance. We have utilized genome-editing technology to functionally knockout some of these rice loci that were negatively regulating rice yield and also its adaptation to various biotic stresses to impart improved agronomic performance. For high yield we have selected genes that are negatively regulating tiller number, panicle size, spikelet number and grain size and weight. Similarly, for the disease resistance we selected to knockout the disease susceptible genes whose expression is essential for disease establishment.

The improved plant architecture is a practical and effective way to increase cereal grain production. We selected the Dense erect panicle 1 (dep1) (Os09g26999), mutation that was named for its erect compact panicle with high spikelet number per unit panicle length and having semi-dwarf plant phenotype with increasing effective tillers to enhance rice grain production. The dep1 is an established rice loss of function allele but gain of agronomic trait under different genetic backgrounds for improved rice grain yield. After introducing the expression cassettes of Cas9 and sgRNA that target to DEP1 allele into MTU1010 indica rice cultivar through agrobacterium-mediated rice transformation and generated several transgenic lines. These transgenic rice lines were analyzed for knockout mutation by sequencing the PCR DNA fragment amplified using the specific primer pair flanking the designated target site in DEP1 allele. The sequence analysis identified various deletion mutations in the DEP1 allele among different transgenic lines. Most frequently less than 10 nucleotides were deleted at the targeted double strand break region, we also observed larger deletions of more than 50 nucleotides. Most of the smaller deletions were observed in single allele mutation whereas, the larger deletions were frequently observed in bi-allele mutation. We found the targeted

nucleotide deletions observed in the DEP1 allele leading to frame shift mutation and premature truncation of coding region. The mutant lines were grown in the field and the phenotype of homozygous T2 plants showed semi-dwarf plant phenotype with increasing effective tillers having Dense erect panicle containing more spikelets. The loss of function of dep1 allele generated through targeted genome editing in MTU1010 indica rice cultivar improved plant architecture and enhanced grain productivity.

Similarly, the targeted nucleotide deletion mutations introduced into rice OsTB1 (Os03g49880) allele using Cas9 based genome editing technology produced more productive tillers in the rice plant. The rice TB1 was extensively characterized for its role in negatively regulating the lateral meristem dormancy, the non-functional allele of TB1 allows the extensive growth of the axillary buds results in increased tiller number. The growth and development of shoot apical and lateral meristems responsible for increasing productive tiller number double in the loss of function of tb1allele generated through targeted genome editing in MTU1010 indica rice cultivar produced more productive tillers and enhanced grain productivity. The pleiotropic effect of increasing tillers may reduce the spike size. To compensate this pleiotropic effect, we propose to pyramid the OsCKX2 (Os08g0509600) knockout mutation in rice. The OsCKX2 encodes for cytokinin oxidase that inactivate the plant hormone cytokinin and negatively regulates the plant growth and cell division. Using the Cas9 endonuclease we introduced targeted deletion mutations in OsCKX2 encoding region of the rice genome. The OsCKX2 knockout rice lines accumulated significant amount of active cytokinin in compared to their wild type rice plants results in improving plant growth and cell division, the increasing rice spike size of OsCKX2 knockout mutant rice produced almost double the number of rice grains compared to their wild type allele in MTU1010 rice cultivar. We are in the processes of introducing knockout mutations in OsGS3 (Os03g0407400), OsGW5 (Os01g25010) and OsTGW (Os06g41850) alleles of MTU1010 rice cultivar to increase rice grain length and weight significantly. The non-functional natural mutant alleles of OsGS3 (Os03g0407400), OsGW5 (Os01g25010) and OsTGW (Os06g41850) were earlier shown to influence the rice grain length and weight significantly in other rice lines.

In addition to the direct yield related traits the resistance to major pathogens is also an important agronomic trait for the sustainable rice productivity. Several genetic loci were identified in rice plants whose expression is essential for disease establishment by the pathogens. Either one

could mutate and/or knockout these disease susceptible alleles to impact the resistance to targeted pathogens. Rice blast is one of the most destructive diseases caused by the fungus, *Magnaporthe oryzae*. Most of the adaptive *M. oryzae* isolates fungus induces a set of rice genes were induced rapidly OsERF922, OsMADS26, OsMAPK5 and OsPLD□1 immediately after infection. These pathogens induced loci in the rice genome that negatively regulates the expression of plant innate defense system and helps in establishment of pathogenesis. The knockdown or knockout of these rice loci conferred blast resistance and prevented *M. oryzae* infection without affecting the rice plant growth and development. We initiated to generate knockout mutations in OsERF922, OsMADS26, OsMAPK5 and OsPLD□1alleles of MTU1010 rice cultivar using Cas9 based targeted gene deletion mutagenesis for durable, pathogen strain independent blast resistance.

The extensively characterized high yielding and/or disease resistant natural mutants fund in the rice germplasm in different genetic backgrounds. Here we showed these mutations could be recreated directly in the elite rice cultivar using the targeted genome technology. Our results showed the selective frame-shift mutation introduced into Osdep1 (Os09g26999) allele in MTU1010 rice cultivar has produced erect compact panicle having high spikelet number per unit panicle length. Similarly, the frame-shift mutation introduced into Ostb1 (Os03g49880) allowed the extensive growth of the axillary buds results in increased tiller number in MTU1010 rice cultivar. These observations suggest that the selective gene knockout mutations generated using targeted genome editing in the elite rice cultivar gave similar phenotype as observe in the natural mutations reported earlier in different genetic backgrounds. The current genome editing methodology could introduce the required combination of mutations directly into any elite rice cultivar without the requirement of any beneficial mutant alleles or the associated time-consuming plant breeding processes for improvement of rice productivity. Therefore, the targeted genome editing is not any different from that which occurs in nature. There is no environmental risk evoked and release of targeted genome edited plants into the environment is as safe as that of traditionally bred plants.

Genome editing Technology for enhancing resistance against fungal and bacterial diseases in crop plants

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Introduction

The importance of plant health has been recognized by United Nations (UN) - an intergovernmental organization, and declared 2020 as an international year of plant health. In total 40 percent of crop plants succumb pre- and post-harvest losses due to pest and diseases and incur a loss of 220 billion USD in trade (IPPC, 2019). Globally plant disease management by chemicals attracted criticism on environmental and trade grounds. In the recent years, Indian farmers faced export rejections due to fungicide residues especially in rice. Breeding for resistance against diseases is laborious and non-durable owing to rapid emergence of new pathotypes and races of economically important plant pathogens of major agricultural crops. The climate change related issues are only compounding the problems in crop production (Zhang *et al.* 2018). Though the genetic manipulations and the consequent modified crops hold good for global food security, the social acceptance is yet another hurdle at the moment. Under this current scenario, the genomics assisted crop breeding by adopting genome editing technologies is very promising. The site-specific targeted modifications in the crop plants to make them tolerant biotic and abiotic stress without any off or non-target effect are worth attempting.

Genome editing is termed as manipulation of the specific gene loci to gain genome modifications, such as insertions, deletions or point mutations (Hala *et al.*, 2018). It is a potential technology which facilitates understanding and treatment of various diseases, and can reveal the genetic and molecular basis pathogenesis and host resistance. Specifically, engineered nucleases help the process of editing the genomes which are divided into two major groups i.e., engineered protein guided nucleases and engineered RNA/DNA guided nucleases (Parichita *et al.*, 2019). Most popularly known three fundamental genome editing technologies are *i. Transcription activator-like effector nucleases (TALENs), ii. Zinc-finger nucleases (ZFNs)* and iii. *A bacterial immune system based clustered regularly interspaced short*

palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) (Gaj *et al.,* 2016). Recently genome editing technology witnessed wide applications in medical research to study of cancer biology (Sánchez-Rivera *et al.,* 2015), human gene therapy (Gori *et al.,* 2015) and also crop improvement through Next Gen-Plant Breeding (NGPB) (Zhang *et al.,* 2017). Currently, in the era of genomics assisted resistance breeding it serves as an important tool to develop new resistant varieties of crop plants specially to impart fungal & bacterial disease resistance to sustain the yield potential. Genome editing can advance the plant breeding without even introducing "controversial" transgenes (Araki *et al.,* 2015) but by targeting the genes for manipulation of those involved in **plant recognition during infection process, upregulation of defence pathways**, **disarming the susceptibility, secretion of antimicrobial compounds** and **silencing of essential pathogen genes** (Ashajyothi *et al.,* 2019). The efficient global policy can make this technology more applicable for the benefit of future agriculture with social acceptance.

Genome editing technologies

Genome editing can be done by both conventional and modern approaches. Under conventional approach random mutations can be created by natural and mutagens induced mutagenesis. However, the efficiency and specificity of the emerging new technologies in the past two decades replaced the age-old techniques because of their off-target effects (Khan, 2019). The initial techniques used to edit the genome evolved from the earlier attempts like nuclease technologies, homing endonucleases, and certain chemical methods. Molecular techniques like mega nuclease, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) emerged as new technologies as they show sequence specific nuclease (SSNs) activity to create double standard breaks (DSB) in the target DNA. But only after the discovery of a bacterial immune system-based technology called clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) the genome editing came into limelight with the ease, efficiency, feasibility, and multi-role application in various research areas. The genome editing technologies for conferring host resistance as a method in crop protection are briefly discussed over here. Modern genome editing techniques broadly divided in to two groups. They are engineered protein guided nucleases and engineered RNA/DNA guided nucleases.

1. **Engineered protein guided nucleases:** Transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) comes under this group. ZFNs are artificial restriction enzymes engineered by fusing zinc finger DNA-binding domain and DNA- cleavage domain where as TALENs are encoded by plant pathogenic bacteria which induces the expression of host plant genes (Schornack *et al.*, 2013) against which plants have developed natural resistance mechanisms. These nucleases induce double-strand breaks at desired loci that can be repaired by error-prone nonhomologous end-joining to yield small insertions and deletions at the break sites (Wood *et al.*, 2011). ZFN technology successfully applied in crop plants like soybean, corn (Shukla *et al.*, 2009), tobacco (Townsend *et al.*, 2009) and Arabidopsis (Osakabe *et al.*, 2010).

1.1. ZFN and TALEN mediated genome editing for disease resistance

Li *et al.* (2012) have developed bacterial blight resistance in rice through TALEN based gene editing process by targeting the Os*SWEET14* gene. In another study TALENs used as gene editor where hexaploid bread wheat gene Ta*MLO* was targeted and developed wheat variety showing heritable resistance to powdery mildew (Wang *et al.*, 2014). Engineering plants to make them more resistant to emerging pathogens needs advanced technologies however, currently both the ZFNs and TALENs are less preferred because of tedious procedure to obtain target specificity and also sometimes toxic to the host cells (Xiong *et al.*, 2015).

2. **Engineered RNA/DNA guided nucleases**: RNA dependent DNA cleavage systems like CRISPR-Cas9, class2 candidate1 CRISPR-Cas9 and RNA dependent RNA cleavage systems like RNAi and Class 2 candidate 2 CRISPR-Cas9 comes under this group. Among various CRISPR based systems, type II CRISPR/SpCas9 system from *Streptococcus pyogenes*, have been considered as versatile genome-editing tool (Hsu *et al.*, 2014). This system is known for a wide variety of potential applications compared with ZFNs and TALENs which is based on its simplicity, efficiency, and low cost, and by its ability to target multiple genes. In recent past till date, many crops such as rice, maize, wheat, soybean, barley, sorghum, potato, tomato, flax, rapeseed, Camelina, cotton, cucumber, lettuce, grapes, grapefruit, apple, oranges, and watermelon have been

edited by this technology. Apart from nutritional aspects and other traits improvement, induction of disease resistance and development of resistant varieties by genome editing is a promising strategy for next generation crop protection. This technology has become a boon to improve resistance to biotic stresses in many important crop plants.

2.1. Engineered RNA/DNA guided nucleases mediated genome editing for disease resistance

Zhang *et al.* (2017) generated *Taedr1* wheat plants by simultaneous modification of the three homoeologs of EDR1 and developed plants resistant to powdery mildew which did not show mildew-induced cell death. Wang *et al.* (2016) enhanced rice blast and bacterial blight resistance by mutagenesis of Os*ERF922* and Os*SWEET13*. Furthermore, Citrus canker another important disease with significant economic losses worldwide, and *CsLOB1* is a susceptibility gene known for citrus canker. Jia *et al.* (2016) modified the CsLOB1 promoter and found canker symptoms were alleviated in Duncan grapefruits and Wanjincheng oranges that had enhanced resistance to citrus canker (Peng *et al.*, 2017). Even the disruption in the coding region of CsLOB1 in Duncan grapefruits, resulted the crop that had no canker symptoms (Jia *et al.*, 2017). Two diseases of tomato viz., powdery mildew (*Leveillula taurica*) and bacterial speck (*Pseudomonas syringae* pv. *tomato*) diseases that causes significant crop losses. Genome editing helped in developing powdery mildew-resistant tomatoes by editing *SlMLO1*, (Nekrasov *et al.* 2017), and bacterial speck-resistant tomatoes were created by disrupting SlJAZ2 (Ortigosa *et al.*, 2019) a co-receptor for coronatine prevented re-opening of stomata to prevent entry of bacteria thus to enhance resistance.

Resistance enhancement through genome editing: Where to look for the candidates

With the advancement in genomics, whole genome data or many crops and important fungal, and bacterial pathogens is already available in public domain. Every host pathogen combination or pathosystem is unique in its mechanism of pathogenesis and susceptibility. Hence, understanding of mechanism behind the susceptibility and resistance in variety is utmost important to select the target for genome editing to enhance the resistance. Genome editing also provides opportunity to do functional genomics studies to find out the key regulatory genes involved in various host pathogen interactions at various steps of their

interaction. In this process, finding and targeting elicitor-receptor combinations in PAMP triggered immunity (PTI); effector triggered immunity and susceptibility (ETI & ETS) can be one of the best options as potential candidate for directed editing.

i. **Plant Immune system associated genes:** Plants have developed an array of sophisticated immune system to negotiate with pathogens. The innate immune mechanisms to suppress pathogens from causing disease are well connected molecular events. The response of plants to pathogen invasion largely by pathogen recognition receptors at the extra and intra cellular level, which then triggers complex signaling pathways (Jones and Dangl, 2006; Andolfo and Ercolano, 2015). When the plant perceives the signals of danger as pathogen-associated molecular patterns (PAMPs) or danger or damage-associated molecular patterns (DAMPs), or effectors or elicitors, prompt the stereotypical defense programme (Wise *et al.*, 2007). The plant innate immune system is based on two distinct but interconnected components, namely the PTI and ETI. The PTI is based on a large number of surveillance receptors: pattern-recognition receptors (PRRs). PRRs are further classified as Receptor like Kinases (RLK), Receptor like Proteins (RLP), Intracellular Soluble Receptors (ISR) and Extracellular Soluble Receptor (ESR). Genes associated with PTI can be a potential target for genome editing mediated defense. ETI is based on Nibblers (NB-LRR receptors) that recognize the presence of highly polymorphic pathogens protein termed as effectors and convey the message of invasion. ETI is further guided by phytohormones that play a fundamental role in regulating plant immune response (Shah, 2003). Genes associated with ETI can be a potential target for genome editing mediated defense. R-genes in plants detect the activities of effectors and induce ETI to protect them from potential invaders (Uehling *et al.* 2017). These are the cognate effector genes for R-gene products that modulate the virulence and avirulence function during compatible and incompatible interactions. Manipulation of targets of these genes (proteins) through genome editing technologies leads to the enhanced resistance. *avrBS2* of *X. campestris* pv. *vesicatoria*; *pthA* of *X. citri*; *pthN* of *X. campestris* pv. *malvacearum*; *avrRPM* of *P. syringae* pv. *maculicola* are some of the avirulence genes which modulate virulence and avirulence function (White *et al.* 2000). Though R- gene based resistance frequently overcome by pathogen induced mutations (Parlevliet, 1993) as it is one of the cheapest crop protection strategy can help the crops for short term from potential threats.

R- gene domains such as NB-LRR, eLRR, and LRR-Kinase super families are ubiquitous in plants and can be potential targets for genome editing. 'Positional cloning' approach for *R* gene cloning from variety of crops or their wild relatives and to rapidly transfer them into elite cultivars promises enhanced defence (McDowell *et al.* 2003). Pepper- *BS2* against bacterial spot of tomato and pepper (*Xanthomonas campestris*) (Tai *et al.* 1999); barley - *Rpg1* gene against stem rust (Horvath *et al.* 2003); tomato- *Ve1* and *Ve2* against verticillium wilt (Kawchuk *et al.* 2001) genes. There are other R-genes such as *PR-1a*, *SAR8.2*, *AP24* which code antifungal proteins, PR-genes and hydrolytic enzymes can also be chosen as targets (Melchers *et al.* 2000).

ii. **Plant Susceptibility (S)-genes:** These genes are essential for compatible plant-pathogen interactions. Knocking out the S-genes in plants will result in loss of function and brings recessively inherited resistance. Studies in grapes, tomato, pea, pepper and barley shows typical class of S-genes, MLO (Mildew Locus O) genetic factors responsible for susceptibility towards powdery mildew which are conserved across plant kingdom and divided into seven clades (Zaidi *et al.* 2018). Among the seven, clade IV (monocot S-genes) and clade V (dicot S-genes) and inactivation of *MLO* resulted into the recessive *mlo* based resistance (Jorgensen, 1992) which otherwise managed by sulphur based synthetic chemical fungicides. Another important study comes from rice where SWEET genes which are also type of S-genes i.e., *OsSWEET11* and *OsSWEET14* responsible for bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) development. Modification in promoter region of these genes helped in developing resistant varieties (Jiang *et al.* 2013). Rice-*OsMPK5* against rice blast and glume blotch (Xie *et al.* 2013); wheat-*TaMLO* and *TaEDR1* against powdery mildew (Shan *et al.* 2013); tomato-*DMR6* against *Pseudomonas syringae*, *Phytophthora capsici*, and *Xanthomonas* spp. (de Toledo *et al.* 2016); citrus -*CsLOB1* against citrus canker (Jia *et al.* 2017); apple-*DIPM-1, 2 & 4* against fire blight (Malnoy *et al.* 2016) are some of the best examples for S-genes that can be a target for resistance breeding.

iii. **Regulators of Systemic Acquired Resistance /Induced Systemic Resistance:** Over expression of genes responsible for development of SAR/ISR in plants can be another target for genome editing. Cao *et al.* (1998) found over expression of essential regulatory

gene *NPR1* confers resistance to the important pathogens *Pseudomonas syringae* and *Peronospora parasitica* in a dosage-dependent fashion. It is well known that *NPR1* overexpression leads to enhanced resistance with no obvious detrimental effect on the plants**.** Dong, 2004 highlighted *NPR1* has multiple roles in defence modulation such as regulation of salicylic acid (SA)-mediated systemic acquired resistance and rhizobacterium-triggered induced systemic resistance, also is involved in crosstalk inhibition of jasmonic acid (JA)-mediated defence responses. Apart from *NPR1* and *TGA* transcription factor, the single-stranded DNA-binding transcription factor *AtWhy1* and the *WRKY70* transcription factor were recently found to be involved in SA-mediated defence and SA-JA crosstalk.

Success stories of enhanced crop resistance by genome editing

Genome editing or modification of plant genes in a targeted manner has yielded desired enhanced resistance in many crops (Table 1).

Table 1. Successful genome editing in crop plants for enhancing disease resistance

Technology used for genetic engineering	Targeted gene	Crop	Resistance against	References
Agrobacterium-mediated transformation	*NPR1*	Tomato	Broad spectrum fungal & bacterial disease resistance	Lin *et al.* 2004
TALENS	*SWEET14*	Rice	Bacterial blight	Li *et al.* 2012
CRISPR/Cas9	*SWEET11*	Rice	Bacterial blight	Zhou *et al.* 2014
TALEN and CRISPR/Cas9	*TaMLO* homoeologs and *TaMLO-A1*	Wheat	Powdery mildew	Wang *et al.* 2014
RNAi	*VvMLO6* *VvMLO7* *VvMLO11&13*	Grapevine	Powdery mildew	Pessina *et al.*, 2016
CRISPR/Cas9	OsERF922	Rice	Rice blast	Wang *et al.* 2016
CRISPR/Cas9	*SlDMR6-1*	Tomato	Bacterial speck	De Toledo *et al.* 2016
CRISPR/Cas9	*Taedr1*	Wheat	Powdery mildew	Zhang *et al.* 2017
CRISPR/Cas9	*CsLOB1*	Orange	Citrus canker	Peng *et al.* 2017
CRISPR/Cas9	*CsLOB1*	Grape fruit	Citrus canker	Jia *et al.* 2017
CRISPR/Cas9	*Xa13 promoter*	Rice	Bacterial blight	Li *et al.* 2019

It is to be noted that the editing of candidate plant genes such as SWEET family, MLO, ERF etc playing a role in pathogen effector triggered susceptibility yielded improved crop resistance in crops like rice, wheat, grapevine. Frequent success stories after 2014 is an indication that CRISPR/Cas9 based GET is well on the way to make big impact in crop disease management strategies in Next Generation Agriculture.

Conclusion

Since the year 2014 onwards, considerable improvement has been observed in crop improvement by utilizing modern genomics tools such as CRISPR/Cas9. With the ever-increasing demand for food grains and rapidly degrading natural resources, conventional agriculture no longer able to feed growing population. Hence, judicious use of genome editing technologies for developing resistant cultivars is an obligation to avoid extensive chemical application and tedious breeding programmes. As it is already evident that cultivars developed with the help of various genome editing approaches have shown both durable broad spectrum and vertical resistance against economically important pathogens. Therefore, future breeding programmes must be designed by taking assistance from genome editing technology (GET) tools to target the key regulators or components of host pathogen interactions to accelerate and enhance the efficiency of resistance breeding.

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Practical

Chapter 1

Single Guide RNA design for CRISPR/Cas9 based genome editing: Principles and tools

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Introduction

Genome editing is the addition, deletion, substitution of nucleotide(s) at a desirable place in the genome, which may result in the knockout, knockin, activation, or inhibition of a particular gene. Various techniques such as clustered regularly interspaced short palindromic repeats (CRISPR) –CRISPR-associated protein9 (Cas9) (CRISPR/Cas9 technology), Zinc finger nuclease (ZFN) and Transcription activator like effector nuclease (TALEN) have been used for genome editing. Among these, the most common technique used nowadays is CRISPR/Cas9 based genome editing.

CRISPR/Cas9 based genome editing is a kind of two-component system of which the first component is single guide RNA (sgRNA) which is a combination of CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA) and the second component is Cas9 enzyme. Designing of sgRNA is a key for successful CRISPR/Cas9 genome editing. A part of the sgRNA is conserved, which is present in every sgRNA while other part needs to be designed according to the target gene. Critical designing of sgRNA is essential for editing of gene; otherwise, it may result in off-target effects. In this chapter, we will discuss various factors that should be considered for designing sgRNA as well as multiple tools used for designing sgRNA.

Principle of sgRNA design

A sgRNA is a combination of crRNA and tracrRNA fused through a linker sequence. The crRNA sequence is usually 20 nucleotides long and specific to the target gene while tracrRNA is constant and can be made *in vitro* from a DNA template or can be chemically synthesized. The basic principle is to design a suitable sgRNA(s) which can target the gene of interest and generate a double-stranded break at the target site. Simultaneously, it should not target any other related sequences in the genome (Figure 1).

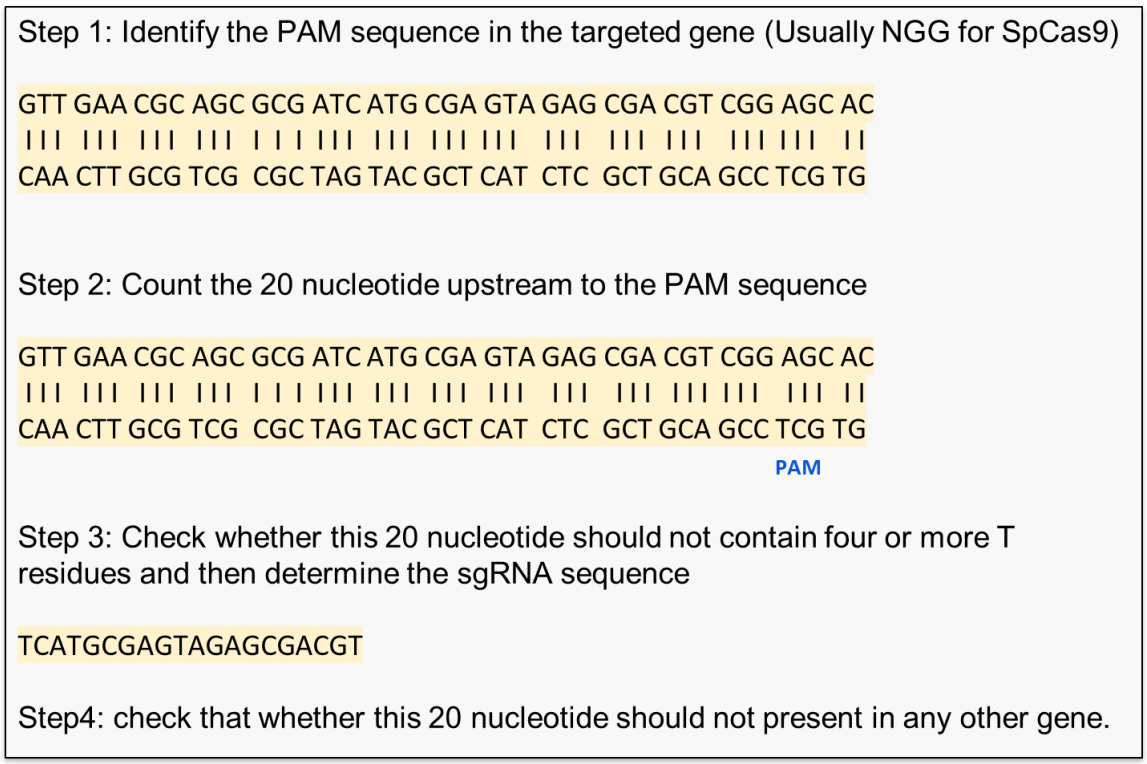

Figure 1. Workflow for sgRNA designing

The critical factors that should be considered for designing of sgRNA:

1. sgRNA can be designed on either strand of the DNA.
2. The length of the sgRNA is usually near 20 nucleotides. Longer sgRNAs are less effective as compared to 20 nucleotides, whereas shorter sgRNAs (17 or 18 nucleotides) show higher specificity but perform less on-target effects.
3. sgRNA sequence should be followed by a protospacer adjacent motif sequence (PAM; 5′NGG3′ *Sp*Cas9) in the genomic DNA.
4. The 20 nucleotide sgRNA and PAM sequence region should not be matched to any other region of the genome. If matches to any, it may result in off-target effects.
5. There should not be four or more continuous thymine nucleotides (TTTT) in the sgRNA sequence because this acts as a terminator for RNA polymerase III promoters.
6. The targeted sgRNA should be only in the exon (not between two exons) if the purpose is to knockout the gene.
7. sgRNA, as far as possible, should be designed from 5′ end of the gene (N-terminal of the protein) for knockout of the gene.

8. If the target is to knockout a family of the gene, then sgRNA should be designed from a consensus region of the gene family.
9. The first nucleotide of sgRNA should be Guanine if the small nucleolar RNA U6 promoter is used and Adenine if U3 promoter is used.

Software and tools used for sgRNA design:

The designing of sgRNA is very critical for successful genome editing. Various tools and softwares are available to design sgRNA. Each of these tools predicts sgRNA based on certain genomes, on-target prediction score, off-target score, and some other parameters (Cui *et al.* 2018; Thomas *et al.* 2019). In table 1, we have summarized various tools used to design sgRNA and finding of CRISPR locus in bacterial genome.

Among these tools used for designing of sgRNA, CRISPR-P and CRISPR-Plant is exclusively used for the plant genomes.

Table 1. List of tools used for designing of sgRNA and finding of CRISPR locus in the bacterial genome

Tool	Link	Reference
CRISPRfinder	http://crispr.u-psud.fr/Server/	Grissa *et al.* 2007
CRISPI	http://crispi.genouest.org	Rousseau *et al.* 2009
CRISPRTarget	http://bioanalysis.otago.ac.nz/CRISPRTarget/crispr_analysis.html	Biswas *et al.* 2013
CRISPR-Plant	http://www.genome.arizona.edu/crispr	Xie *et al.* 2014
CRISPR-P	http://cbi.hzau.edu.cn/cgibin/CRISPR2/ CRISPR	Lei *et al.* 2014
E-CRISP	http://www.e-crisp.org/E-CRISP	Heigwer *et al.* 2014
GT-scan	http://gt-scan.braembl.org.au/gt-scan	Brien and Bailey. 2014
CRISPRdirect	http://crispr.dbcls.jp	Naito *et al.* 2015
sgRNAcas9	http://www.biootools.com/col.jsp?id=103	Xie *et al.* 2014
CHOPCHOP	https://chopc hop.rc.fas.harvard.edu/	Labun *et al.* 2016
sgRNA Scorer 2.0	https://crispr.med.harvard.edu/sgRNAScorerV2/	Chari *et al.* 2017
CRISPR Multi Targeter	http://www.multicrispr.net/	Prykhozhij *et al.* 2015
GuideScan	http://www.guidescan.com/	Perez *et al.* 2017

Detailed procedure of sgRNA design

Example of sgRNA design using CRISPR-P software

1. Go to the CRISPR-P website http://crispr.hzau.edu.cn/CRISPR2/ homepage.
2. Go to the **start design** option on the website homepage and select various options from the page such as type of PAM sequence, snoRNA promoter, RNA scaffold sequence, guide sequence length, target genome, Locus Id, position or sequence etc. (Figure 2 & 3)

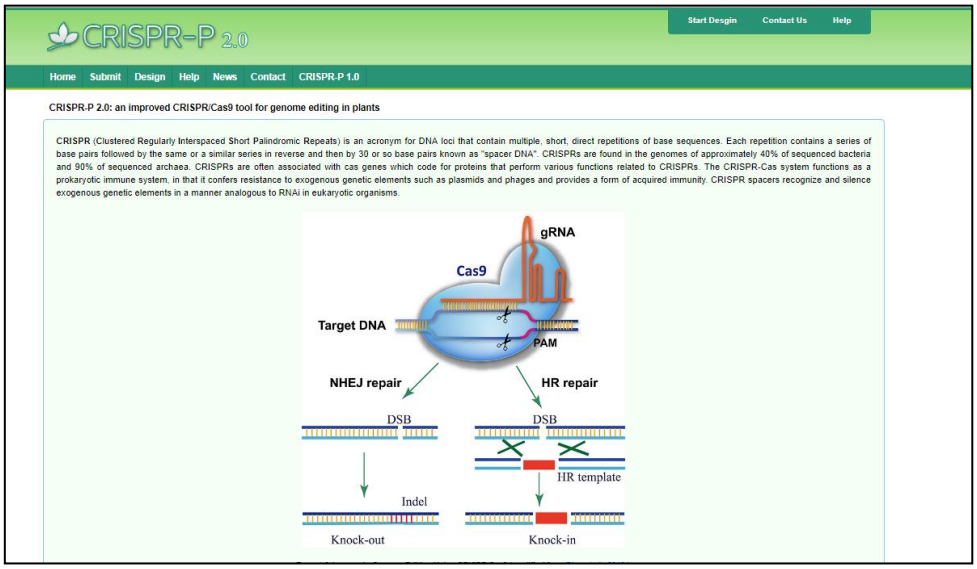

Figure 2. CRISPR-P 2.0 screenshot illustrating home page of CRISPR-P 2.0

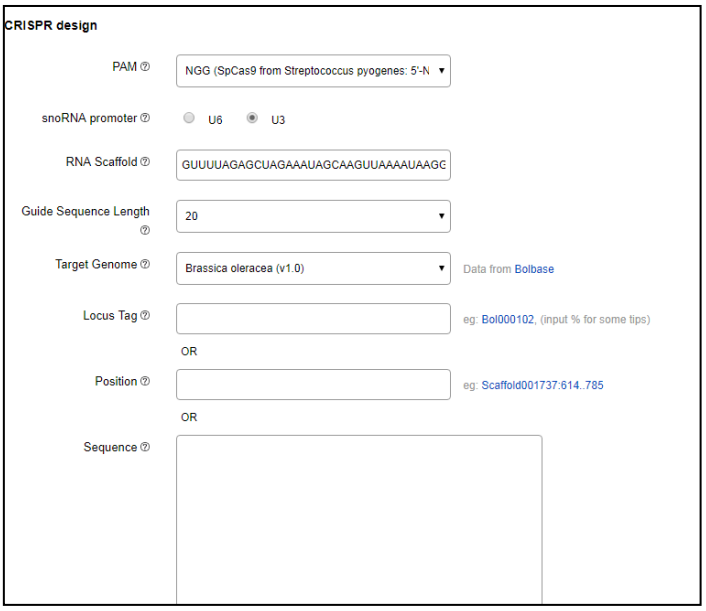

Figure 3. CRISPR-P 2.0 homepage showing various parameters related to CRISPR design such as PAM sequence, small nucleolar RNA (snoRNA) promoter, RNA scaffold, guide sequence length, target genome sequence, locus tag, position, and sequence.

3. Select the target genome and paste the gene sequence or Locus ID or position of the gene and click on the submit option (Figure 4).

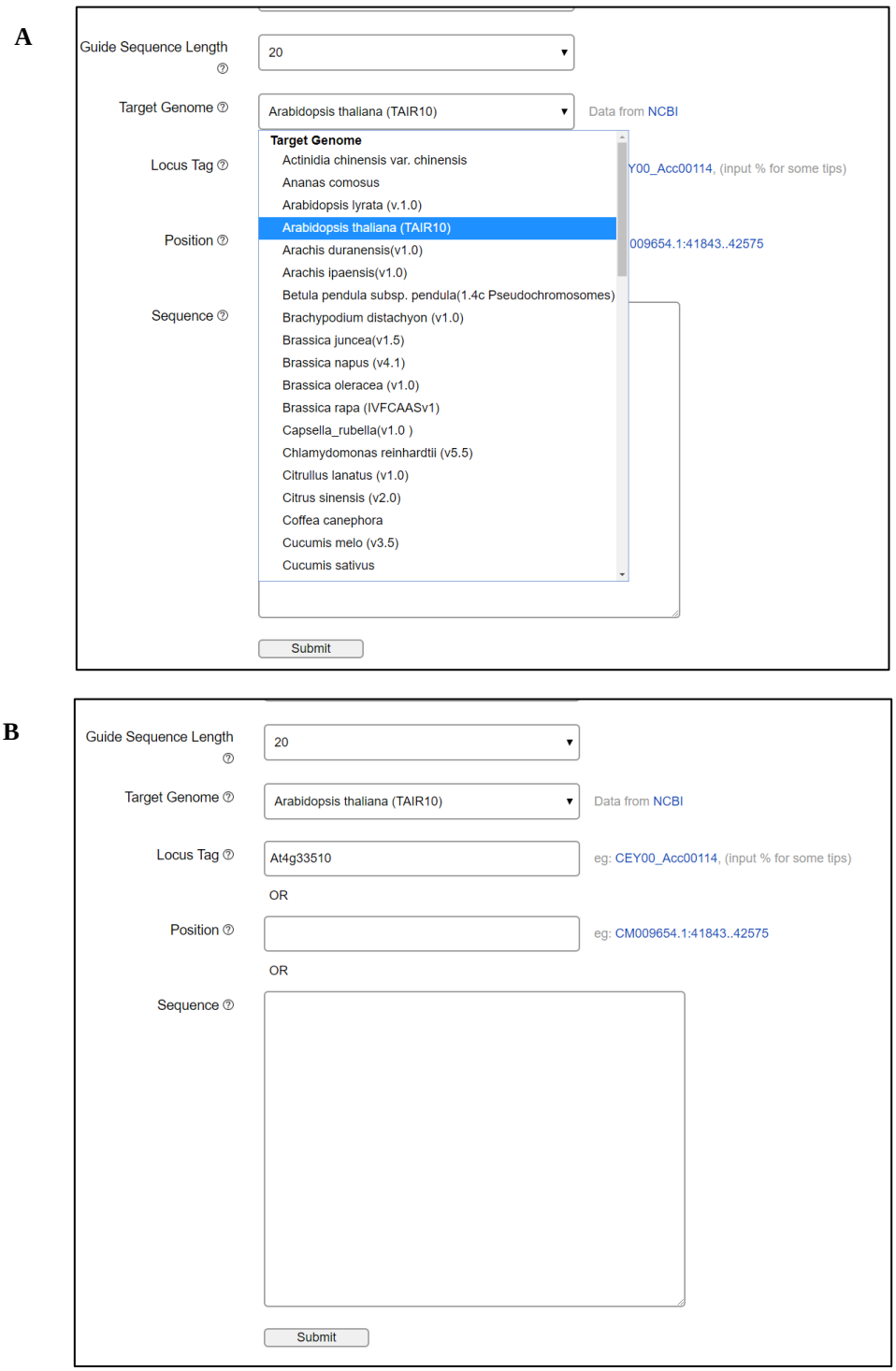

Figure 4. CRISPR-P 2.0 screenshot illustrating A) various target genome B) Locus tag of gene or position or sequence of the gene

Results

After the search button, it will give multiple sgRNA and their sequence along with various other information such as the region of the gene, strand position, on-target score, GC %, off-target score (Figure 5&6).

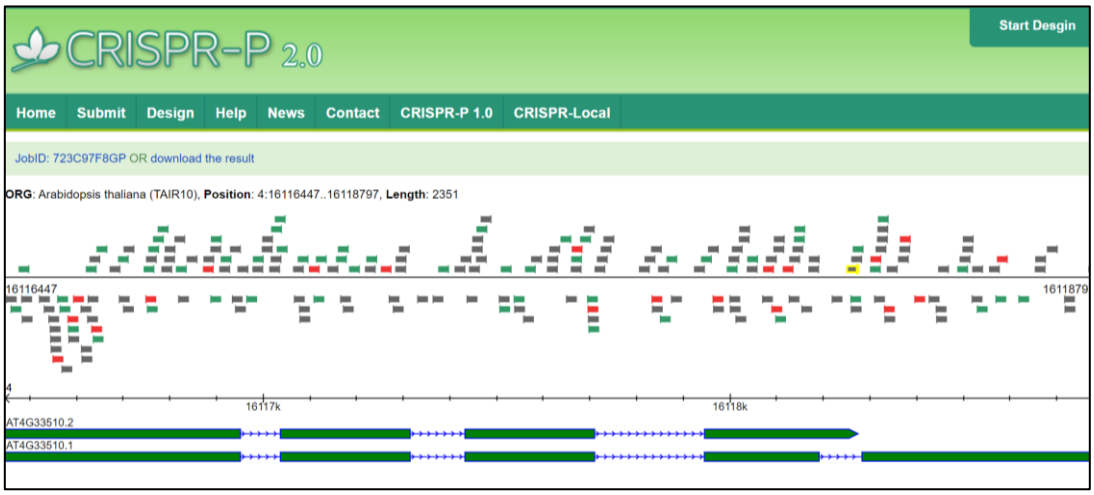

Figure 5. CRISPR-P2.0 screenshot illustrating various sgRNA selected for the target gene, their position, and strand of DNA

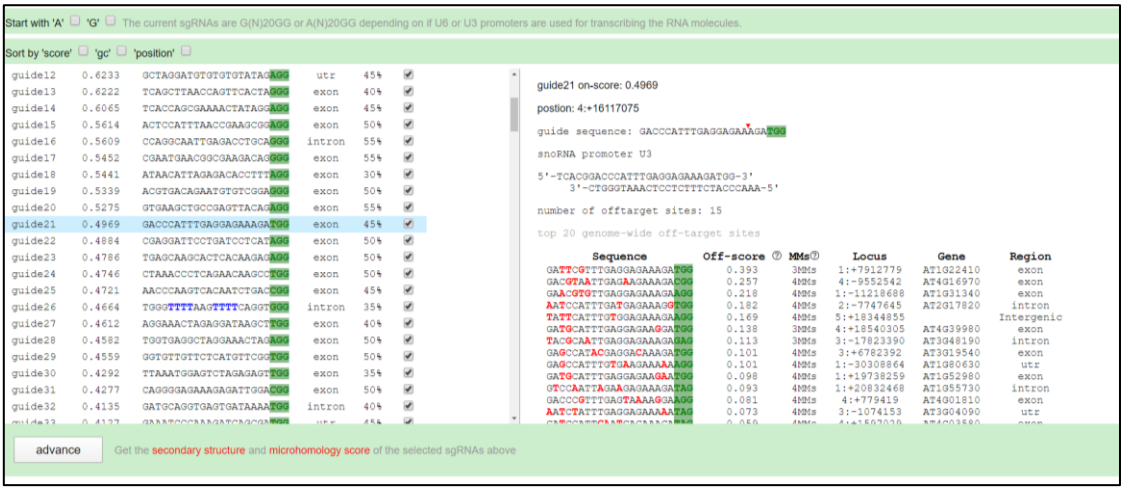

Figure 6. Various sgRNA sequence, on-target score, GC%, the region of the genome (exon/intron/UTR), number of off-targets, off-target score, and other information.

Conclusion

CRISPR/Cas9 technology is becoming very popular day by day due to its easy to use, cheap, highly efficient results. In this present chapter, we have discussed various fundamental principles for sgRNA design. At present, many tools are available for sgRNA design. The main aim of each tool is to design the optimum sgRNA, which can target the gene based on position and high efficiency. Although many tools are available for designing sgRNA, there is no single

tool, which can fulfill everything. Further, sgRNA designed from any tool needs to be confirmed by the *in-vitro* cleavage assay of sgRNA.

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Chapter 2 *In vitro* cleavage of target DNA using crRNA:tracrRNA:Cas9 ribonucleoprotein (RNP) complex

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Principle and Theory

Among the different genome engineering tools, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein (Cas9) (CRISPR/Cas9) system endonuclease (CRISPR/Cas9) has become most relevant due to its inexpensive easy to use nature, preciseness and robustness. CRISPR/Cas9 system consists of *Streptococcus pyogenes* derived Cas9 endonuclease and an RNA duplex consists of a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). Researcher replaced such duplex RNA by a synthetic single guide RNA (sgRNA) module that mimics the crRNA: tracrRNA duplex.

The crRNA has two parts, one consists of the 16–22 nucleotides deriving from 3' end of the repeat sequence and the second part consists of 20 nucleotides complementary to the DNA strand of protospacer (target site), which guides Cas9 to the DNA target. Preceeding to the protospacer sequence, there is a protospacer adjacent motif (generally a 5'NGG3'), which guides the Cas9 to bind with the DNA. The tracrRNA is partially complementary to crRNA and required for crRNA maturation and DNA cleavage by Cas9. The Cas9 is a large protein with two nuclease domains, RuvC-like domain, which cleaves the non-complementary DNA strand and a HNH (His-Asn-His)-like domain, which cleaves the complementary DNA strand. When the crRNA:tracrRNA duplex bind with Cas9 it changes its configuration and become active. Activated Cas9 search in the DNA for a putative PAM sequence. Upon matching with the PAM followed by the complementary sequence of crRNA, Cas9 cause local unwinding of dsDNA followed by a double stand DNA break. The efficacy of crRNA depends upon the Tm and GC content.

In order to test the functionality and relative efficiency of CRISPR/Cas9 system, *in vitro* cleavage of DNA can be carried out in one step by CRISPR/Cas9 ribonucleoprotein complex. Several companies like Integrated DNA Technology (IDT), USA; Thermo Scientific and Takara-Contech developed an easy in vitro assay system to evaluate the efficacy of crRNA before developing a construct.

Here we will discuss about the Alt-R CRISPR-Cas9 System of IDT that is based on the natural *S.* pyogenes CRISPR RNA system and utilizes the optimized crRNAs and tracrRNAs that were shortened to 36 and 67 nucleotides, respectively.

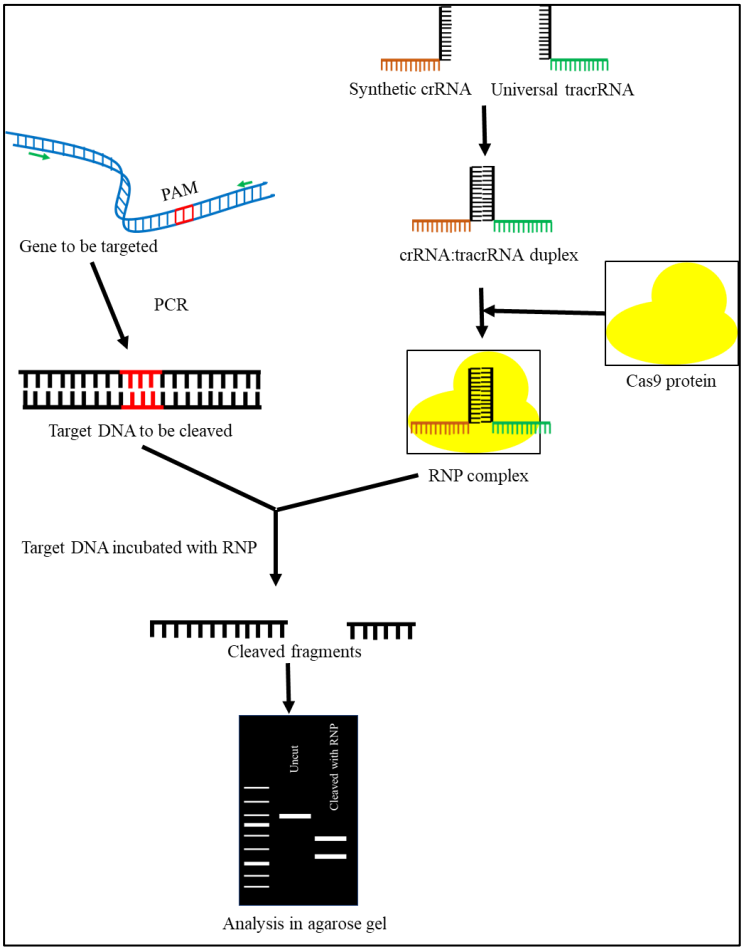
Principally the procedure has three steps:

i) Development of target DNA
ii) Development of crRNA:tracrRNA:Cas9 RNP complex
iii) Invitro cleavage of target DNA by the RNP complex

Target DNA could either be a synthetic dsDNA molecule, or PCR amplified fragment, or cloned plasmid. The crRNA could be commercially synthesized. The universal tracrRNA and Cas9 purified protein are available commercially. An RNP complex can be prepared and when incubated with target DNA, it can cleave the DNA. This protocol demonstrates a method to experimentally validate the activity of CRISPR guide RNA before applying the technique in practical application.

Illustrations-Flow chart/drawings:

Below a schematic representation depicts the principal of the process:



Materials required:

i. Alt-R CRISPR-Cas9 crRNA (To be synthesized)
ii. Alt-R CRISPR-Cas9 tracrRNA [IDT (cat # 1072532, 1072533, 1072534)]
iii. Alt-R S.p. Cas9 Nuclease V3 [IDT (cat # 1081058, 1081059)]
iv. Nuclease-Free Duplex Buffer [IDT (cat # 11-05-01-12)]
v. Nuclease-Free IDTE, pH 7.5 (1X TE solution) IDT (cat # 11-01-02-02)
vi. Nuclease-Free Water [IDT (cat # 11-04-02-01)]
vii. DNA substrate containing the target sequence (PCR amplified/synthetic oligos/cloned DNA)
viii. 10X Cas9 Nuclease Reaction Buffer (See composition at the end)
ix. 1X PBS (See composition at the end)
x. Proteinase K (Molecular biology grade)

Detailed protocol:

a) Prepare the double-stranded DNA template as cleavage substrate

- Template could be linearized plasmid, purified PCR products, duplexed synthetic oligos. Final concentration required for template: For linearized plasmid (2000+ bp) : 1-2 nM, for PCR product (100-500 bp): 5-50 nM, for PCR product (500-2000 bp): 2-5 nM. Resuspend or dilute the DNA substrate in Nuclease-Free Water to your final concentration.
- Template should contain a 20 nt guide sequence, followed by the Cas9 PAM site (NGG). The guide sequence should match the target-specific crRNA that will be used for RNP preparation and digestion.
- Ensure you are using a 10:1 molar ratio of Cas9 RNP:DNA substrate to obtain the best cleavage efficiency.

b) Create the crRNA:tracrRNA duplex

- After identifying a protospacer sequence, commercially synthesize crRNA and procure universal tracrRNA and Cas9 protein.
- Resuspend synthesized crRNA and universal tracrRNA in IDTE buffer to a final concentration of 100 µM.
- Mix the two RNA oligos in equimolar concentrations in a sterile microcentrifuge tube to a final duplex concentration of 10 µM:

Component	**Amount (µL)**
100 µM Alt-R CRISPR-Cas9 crRNA	1
100 µM Alt-R CRISPR-Cas9 tracrRNA	1
Nuclease-Free Duplex Buffer	8
Total	10

- Heat the duplex at 95°C for 5 min.
- Remove from heat and allow to cool to room temperature (15–25°C).

c) Create the RNP complex

- Combine the crRNA:tracrRNA duplex and Cas9 enzyme in equimolar amounts.

Component	**Amount (µL)**
10 µM Alt-R crRNA:tracrRNA complex (from previous step)	10
Alt-R S.p. Cas9 Nuclease 3 NLS (62 µM stock)	1.6
1X PBS	88.4
Total	100

- Incubate 5–10 min at room temperature for optimal formation of the RNP complex.

d) Perform the *in vitro* digestion reaction and visualization

- Assemble the reaction at room temperature (15–25°C).

Component	**Amount (µL)**
10X Cas9 Nuclease Reaction Buffer	1
1 µM Cas9 RNP	1
50 nM DNA substrate	1
Nuclease-Free Water	7
Total	10

- Incubate the reaction at 37°C for 60 min.
- Add 1 µL Proteinase K (20 mg/mL) to the reaction, then incubate the mixture at 56°C for 10 min to release the DNA substrate from the Cas9 endonuclease.
- Analyze the digestion by agarose gel electrophoresis

Results and Conclusion

We will see the PCR amplified fragment is cleaved into two as in agarose gel two fragments will be generated (see the schematic diagram figure).

References

1) In vitro cleavage protocol of IDT: Web resource http://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/protocol/alt-r-crispr-cas9-protocol-in-vitro-cleavage-of-target-dna-with-rnp-complex.pdf?sfvrsn=88c43107_2

2) Mehravar, M. *et al.*, 2019. In Vitro Pre-validation of Gene Editing by CRISPR/Cas9 Ribonucleoprotein. Avicenna J Med Biotechnol. 11(3): 259–263.

Chemical preparation and buffer composition:

1. 10X Cas9 Nuclease Reaction Buffer:

- 200 mM HEPES
- I M NaCl
- 50 mM MgCl(2)
- 1 mM EDTA, pH 6.5 at 25°C

2. For 1 liter of 1X PBS, prepare as follows:

- Start with 800 ml of distilled water:
- Add 8 g of NaCl.
- Add 0.2 g of KCl.
- Add 1.44 g of Na_2HPO_4.
- Add 0.24 g of KH_2PO_4.
- Adjust the pH to 7.4 with HCl.
- Add distilled water to a total volume of 1 liter.

Chapter 3

In-vitro cleavage assay of gene/DNA to check the efficiency of sgRNA

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Principle

Sequencing and editing of genes are nowadays a routine practice in the modern biological era. Among various available genome editing technologies, CRISPR/Cas9 has become widely used because of its robustness, simplicity, efficiency, and accuracy. To demonstrate this technology, single guide RNA (sgRNA) and Cas9 enzyme are required. The sgRNA is a combination of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), which can be combined through the linker molecule. The sgRNA (specific to the target site) along with cas9 enzyme form a ribonucleoprotein (RNP) complex, which makes double-stranded breaks at the target site of the genome. This further leads to the activation of cellular repair machinery of the cell causes insertion or deletions, which ultimately leads to disruption of the target gene.

This sgRNA can be generated through *in-vitro* transcription or chemical synthesis, hence, avoiding the cloning processes, which makes validation of CRISPR reagents faster. In CRISPR/Cas9 technology, one gene can be edited by more than one sgRNA. Hence, before editing genes *in-vivo*, the specificity and efficiency of sgRNAs to edit the target genes can be checked *in-vitro* by Cas9 mediated cleavage assay. This assay validates that which sgRNA is best suitable to edit the gene(s) *in-vivo*. This assay is a rapid, convenient, and efficient way to confirm the efficiency of sgRNA.

Materials required

Target specific oligo(s), Target DNA template, Nuclease-free water, RNase-free tubes (0.2, 0.5 and 1.5 ml volume), Microcentrifuge, Thermocycler/37°C heat block/incubator, NanoDrop 2000 (Thermoscientific), sgRNA synthesis kit *S. pyogenes* (NEB# E3322S), sgRNA purification kit (Zymo Research #R1017), Cas9 nuclease, *S. pyogenes* (NEB #M0386), Gel electrophoresis unit, Gel loading dye

Protocol

1. PCR amplification and purification of the gene of interest

Primers pair will be used to amplify the gene of interest from the genomic DNA of the targeted species. The reactions will be set up as given below.

REAGENT	AMOUNT
Genomic DNA	3.0 µl
High fidelity Phusion buffer (5X)	10.0 µl
dNTPs (10mM)	2.0 µl
Forward primer (10 µM)	2.0 µl
Reverse primer (10 µM)	2.0 µl
Phusion DNA polymerase (2 U/µl)	0.5 µl
Nuclease free water	30.5 µl
Total	50.0 µl

The amplification will be performed in a thermocycler with the following amplification conditions.

Step	Temp and Time
Step 1 Initial Denaturation	98°C 30 sec
Step 2 Denaturation	98°C 10 sec
Step 3 Primer annealing	55°C- 65°C as per Tm of the primer for 30 sec
Step 4 Primer extension	72°C time as per the fragment length
Step 5 Repeat steps 2 to 4 X35	
Step 6 Final extension	72°C 7 min
Step 7 Hold ∞	

Checking and purification of PCR product

About 5 µl of PCR amplification product will be mixed with 1 µl of 6X loading dye and electrophoresed in 1.0% agarose gel. The gel will be stained with 0.1% ethidium bromide, and 1kb DNA size marker will be used for estimating the size of the amplicons. The gel will be documented using any gel documentation system. After checking the specificity of the PCR product in the gel it will be purified using any gel purification kit.

2. Primer designing for *in-vitro* sgRNA synthesis

Target-specific oligos are designed as follows:

1. Select 20 nucleotide sgRNA sequence (not including the PAM (NGG) sequence) to target the gene of interest.

2. Check input sequence for the presence of "G" at the 5´ end.

   If there is no "G'" at the 5´ end, add one "G" (making it a total of at least one G at the 5´ end).

3. To the 5´ end; append T7 promoter sequence: TTCTAATACGACTCACTATA

4. To the 3´ end; append 14 nucleotide overlap sequence: GTTTTAGAGCTAGA

5. Check the complete oligo sequence: 5´ TTCTAATACGACTCACTATA **G**AACCAAACATTTCGCTTCCGTTTTAGAGCTAGA 3´

Example

1. Select the target-specific DNA sequence (we recommend using a target DNA selection program):

Example: **G**AACCAAACATTTCGCTTCCAGG

The PAM sequence (NGG, underlined) is required for Cas9 recognition of the target sequence and is NOT part of the sgRNA sequence. Remove these three nucleotides:

5´ TGCAACCTTCATTTCCCTGC

2. Check for "G" at the 5´ end of the target sequence (at least one G is necessary for transcription). If no G is present, add one G: 5´ **G**AACCAAACATTTCGCTTCC 3´

3. To the 5´ end, append T7 promoter sequence (shown in red): 5´ TTCTAATACGACTCACTATA**G**AACCAAACATTTCGCTTCC 3´

4. To the 3´ end, append the 14 nucleotide overlap sequence (shown in blue): 5´ TTCTAATACGACTCACTATA**G**AACCAAACATTTCGCTTCCGTTTTAGAGCTAGA 3´

This represents the final oligo sequence, which needs to be synthesized.

The sequence of the *S. pyogenes* Cas9 Scaffold Oligo [provided as a component of the EnGen 2X sgRNA Reaction Mix] is as follows (5´ to 3´, overlap in blue):

5´AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC 3´

The overlapped oligos:

5′ TTCTAATACGACTCACTATAGAACCAAACATTTCGCTTCCGTTTTAGAGCTAGA---------

3′ ---------- ---------- ---------- ---------- ---------- CAAAATCTCGATCTTTATC

---------- ---------- ---------- ---------- ---------- ---------- - 3′

GTTCAATTTTATTCCGATCAGGCAATAGTTGAACTTTTTCACCGTGGCTCAGCCACGAAA A5′

The dsDNA product following fill in by the DNA polymerase:

5′ TTCTAATACGACTCACTATAGAACCAAACATTTCGCTTCCGTTTTAGAGCTAGAAATAG

3′ AAGATTATGCTGAGTGATATCTTGGTTTGTAAAGCGAAGGCCAAAATCTCGATCTTTAT

CCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT TT

GTTCAATTTT ATTCCGATCA GGCAATAGTT GAACTTTTTC ACCGTGGCTC AGCCACGAAAA

In this example, the final sgRNA sequence would be:

5′GAACCAAACAUUUCGCUUCCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU 3′

3. *In-vitro* sgRNA synthesis:

sgRNA can be synthesized using any sgRNA synthesis kit available with different manufacturers. Here in this protocol, we will be using sgRNA synthesis kit *S. pyogenes* (NEB# E3322S).

It is strongly recommended to wearing gloves and using nuclease-free tubes and reagents while synthesizing sgRNA. Reactions should be assembled in microcentrifuge tubes or PCR strip tubes.

1. Arrange ice and thaw EnGen 2X sgRNA reaction mix and target-specific oligo (stock 1µM, given in section 2 of this chapter). Mix and pulse-spin each component in a microfuge prior to use. Store enzyme mix on ice but assemble reaction at room temperature.

2. Assemble the reaction at room temperature in the order listed. Avoid master mixes, and add the enzyme last to each reaction:

REAGENT	AMOUNT
Nuclease-free-water	3.0 µl
EnGen 2X sgRNA Reaction Mix	10.0 µl

Target specific Oligo (1 µM)	5.0 µl
EnGen SgRNA Enzyme Mix	2.0 µl
Total Volume	20.0µl

3. Mix thoroughly and pulse-spin in a microfuge. Incubate at 37°C for 30 minutes.

4. Transfer reaction to ice.

5. For DNase treatment bring volume to 50 µl by adding 30 µl of nuclease-free water and add 2 µl of DNase I (RNase-free, provided), mix and incubate at 37°C for 15 minutes.

6. Proceed with the purification of sgRNA and then analysis by gel electrophoresis.

4. Purification of sgRNA:

Spin columns will remove proteins, salts, and most unincorporated nucleotides. Please ensure that spin columns are compatible with the size of sgRNAs (~100 nucleotides) and expected RNA yields (4–25 µg). Follow manufacturer's instructions. Here in this protocol, we have used RNA Clean & Concentrator™-25 (Zymo Research, CAT #R1017, #R1018) for purification of sgRNAs.

5. Quantitation by UV Light Absorbance

sgRNA concentration following purification can be determined by measuring absorbance at 260 nm on NanoDrop®. Usually, this kit yields 4–25 µg of sgRNA.

***6. In vitro* digestion of gene of interest with Cas9 Nuclease, *Streptococcus pyogenes* (M0386)**

In-vitro digestion can be done using any Cas9 nuclease available with different manufacturers. Here in this present protocol, we have used Cas9 nuclease from NEB *Streptococcus pyogenes* (M0386).

It is strongly recommended to wear gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 30 µl but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

It is essential to keep the molar ratio of Cas9 and sgRNA per target site at 10:10:1 or higher to obtain the best cleavage efficiency. A calculator for this purpose can be found at https://nebiocalculator.neb.com/#!/ssrnaamt.

Procedure:

1. Assemble the reaction at room temperature in the following order:

Component	Volume
Nuclease-free water	20.0 µl
10X Cas9 Nuclease Reaction Buffer	3.0 µl
sgRNA (300nM)	3.0 µl (30 nM final)
Cas9 Nuclease (1µM)	1.0 µl (~30 nM final)
Pre-incubate for 10 minutes at 25^{0}C	
Substrate DNA (30nM)	3.0 µl
Total	30.0 µl

2. Mix thoroughly and pulse-spin in a microfuge.
3. Incubate at 37°C for 15 minutes.
4. Add 1 µl of Proteinase K to each sample, Mix thoroughly and pulse-spin in a microfuge.
5. Incubate at room temperature for 10 minutes.
6. Proceed with fragment analysis.

Results:

1. Analysis of sgRNAs by gel electrophoresis:

Assessment of sgRNA quality and length can be evaluated by gel electrophoresis. Gels should be run under denaturing conditions to avoid the formation of secondary structure. Mix an aliquot of sgRNA with RNA Loading Dye and load in agarose gel. Further, this can be visualized by staining the gel with ethidium bromide and document with gel documentation system (Figure 1).

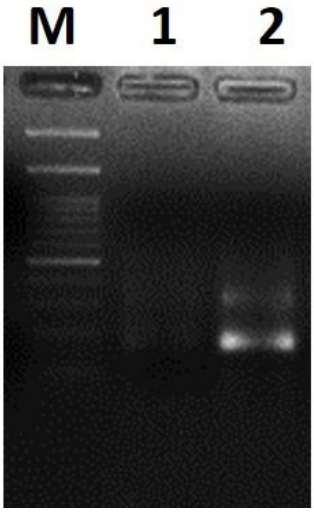

Figure 1. Denaturing agarose gel picture showing the presence of sgRNA, M-100 bp ladder, 1 and 2 are the two different sgRNAs synthesized.

2. *In-vitro* cleavage assay

As shown in Figure 2 lane 4 ribonucleoprotein complex (sgRNA+Cas9 enzyme) is able to creat double-stranded break, which results in the cleavage of gene of interest. This clearly indicates that the cleavage efficiency of this selected sgRNA is very higher.

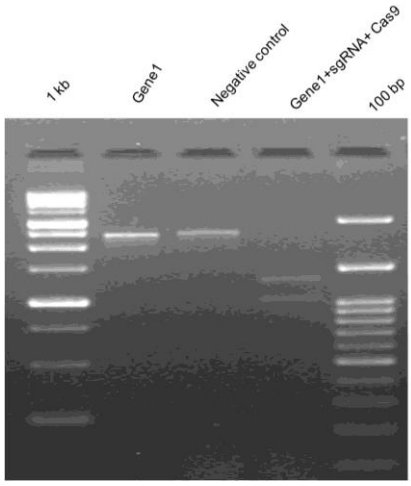

Figure 2. *In-vitro* cleavage assays of Gene1 using sgRNA and Cas9 enzyme.

Conclusion

The designed oligo as per section 2 is able to synthesize the sgRNA, which is able to cleave the target gene *in-vitro* efficiently. This assay is highly useful to check the specificity and efficiency of designed sgRNA.

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Chapter 4 Vector design for CRISPR/Cas9

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Development of CRISPR/Cas9 Vector

Successful genome editing using CRISPR/Cas9 requires binary vector containing sgRNA specific for target genomic region, cas9 gene, promoters for expressing cas9 and sgRNA, terminator for sgRNA, and cas9 gene. Several CRISPR/Cas9 vectors are available at Addgene, which is a non-profit plasmid repository (www.addgene.org).

The first and foremost requirement of the CRISPR/Cas9 vector is the 20 nucleotide sgRNA sequence. Various principles and tools required for designing of sgRNA have been discussed in the earlier chapter.

As CRISPR-Cas9 technology is a two-component system where along with sgRNA, an endonuclease Cas9 is also required. Cas9 is a DNA endonuclease that catalyzes the site-specific cleavage of double-stranded DNA in the presence of sgRNA. As the Cas9 gene is prokaryotic in origin, so for higher expression in eukaryotes, its codon optimization is required. Thus, various codon modified Cas9 has been developed. Initially, it was modified according to human codon usage. Further, it was also altered according to plant codon usage to express in monocots and dicots. In rice, human codon-optimized Cas9 results in 2.5% editing efficiency, whereas plant codon-optimized Cas9 results in 15% editing efficiency (Xu *et al.*, 2014). Therefore, codon optimization of Cas9 is required to increase the editing efficiency.

For achieving the optimal expression of both Cas9 and sgRNA, they should be placed under a suitable promoter. The sgRNA is small in size and usually expressed from RNA polymerase III promoters while Cas9 expressed from RNA polymerase II promoter. In dicots, usually, CaMV 35S promoter is preferred while in monocots, both CaMV 35S and ubiquitin promoters can be used. For expression of sgRNA, usually small nucleolar RNA gene promoters such as U3 or U6 are used. U3 promoter preferred adenine (A), and U6 promoter preferred guanine (G) for transcription start site. In dicots, mostly U6 promoter is generally used, while in monocots, both U6 and U3 promoters are used.

Apart from the promoter, transcription termination sequences are also very important components of the CRISPR/Cas9 vector. Cas9 gene is usually terminated by the nopaline synthase terminator region ($_T$NOS) or octopine synthase terminator region ($_T$OCS). While for

sgRNA stretch of four to six thymine residues (TTTTTT) are required as the terminator sequence.

Materials required:

sgRNA Cas9 cloning vector

Standard oligos

Annealing buffer (NEB)

BbsI (NEB/fermentas)

T4 DNA ligase (NEB or Fermentas)

T4 polynucleotide kinase

Alkaline phosphatase

Microcentrifuge, Thermocycler/37°C heat block/incubator, NanoDrop 2000 (Thermoscientific), Gel electrophoresis unit, Gel loading dye

PCR purification kit

Protocol for CRISPR/Cas9 vector development

sgRNA can be cloned in the CRISPR/Cas9 vector through overlap extension PCR or through restriction digestion and ligation mediated approach.

I) Cloning of sgRNA in sgRNA Cas9 cloning vector through ligation of sgRNA

Vector digestion

1. Digest 1.0 µg of sgRNA Cas9 cloning vector with *Bbs*I for 1 hour at 37°C (Overnight is recommended for completed digestion):

Plasmid	1µg (X µl)
Fast digest BbsI	1µl
Fast alkaline phosphatase (Fermentas)	1µl
10X fast digest buffer	2µl
Nuclease free water	Volume to make 20µl

2. Gel purify digested vector and elute in elution buffer or nuclease-free water (measure the concentration by Nanodrop).

Phosphorylation reaction and oligo annealing

1. Design and synthesize two oligos (standard de-salted oligo is sufficient):
add TGGC at the 5' end of forward oligo, and AAAC at the 5' end of reverse oligo.

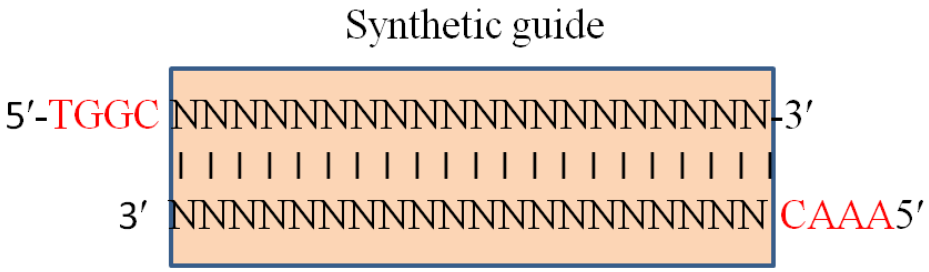

Figure 1. Schematic representation of two designed oligos and their annealing.

2. Preparation of reaction for phosphorylation and annealing of oligos:

Oligo1 (100µM)	1µl (from stock solution)
Oligo2 (100µM)	1µl (from stock solution)
10X annealing buffer	1 µl
Nuclease free water	6.5 µl
T4 polynucleotide kinase (NEB)	0.5 µl

Anneal in a thermocycler using the following parameters:

37 °C for 30 minutes
95 °C for 5 minutes
And then ramp down to 25 °C @ 5°C/min

Primer dilution

1µl of above annealed reaction is diluted with 199 µl of nuclease-free water

Ligation and transformation

1. Set up ligation reaction as follows and incubate at room temperature (or 22C) for one hour (or more):

Digested sgRNA Cas9 vector	X µl (~50 ng)
Diluted Annealing oligo duplex	1µl
10X T4 DNA ligase buffer	1µl
T4 DNA ligase	0.5 µl
Nuclease free water	Volume to make 10µl

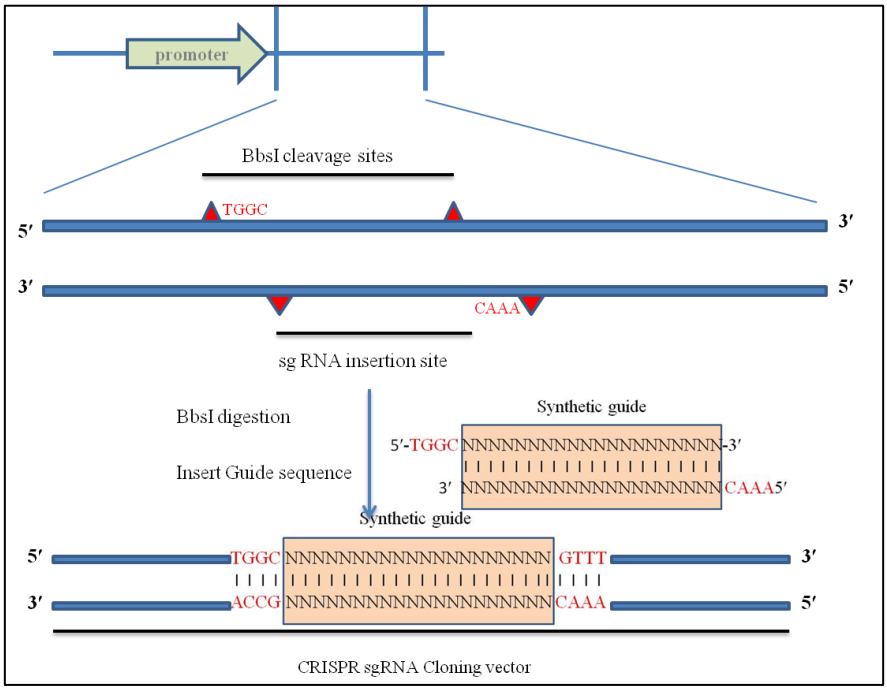

Figure 2. Diagrammatic illustration of cloning of sgRNA in CRISPR/Cas9 vector.

The ligated product is transformed in *E. coli.* DH5α competent cells discussed in next chapter.

II) Overlap extension PCR strategy

Step1. Design four primers as given in figure 3 and order the primers. To clone the sgRNA into sgRNA cloning vector, a total of 4 primers are required two of which are sgRNA-specific (R1 and F2)

F1: AAAGAATTCNNNNNNNNNNNNNNNNNNNN
R1:(N20)NNNNNNNNNNNNNNNNNNN
F2: RC(N20)NNNNNNNNNNNNNNNNNNN
R2:AAAGGATCCNNNNNNNNNNNNNNNNNNN

(N20)- new sgRNA sequence
RC(N20)- reverse complement of new sgRNA sequence

Step 2: Perform two PCR reactions one using F1+R1 primers and second using F2+R2 primers.

REAGENT	**AMOUNT**
Genomic DNA	3.0 µl
High fidelity Phusion buffer (5X)	10.0 µl
dNTPs (10mM)	2.0 µl
F1 primer (10 µM) / F2 primer (10 µM)	2.0 µl
R1 primer (10 µM)/ R2 primer (10 µM)	2.0 µl

Phusion DNA polymerase (2 U/µl)	0.5 µl
Nuclease free water	30.5 µl
Total	50.0 µl

The amplification will be performed in a thermocycler with the following amplification conditions.

Step	**Temp and Time**
Step 1 Initial Denaturation	98°C 30 sec
Step 2 Denaturation	98°C 10 sec
Step 3 Primer annealing	55°C- 65°C as per Tm of the primer for 30 sec
Step 4 Primer extension	72°C time as per the fragment length
Step 5 Repeat steps 2 to 4 X35	
Step 6 Final extension	72°C 7 min
Step 7 Hold ∞	

Step3: Gel purifies the PCR products from both the PCR reactions and perform a new PCR reaction using primer F1 and R2 (follow the above-mentioned protocol for primer combination F1 and R2). Gel purification is essential in this case to remove the original plasmid DNA template.

Step5: Digest the PCR reaction and plasmid DNA using *EcoR*I and *Bam*HI restriction enzymes.

Plasmid/PCR product	500 ng (X µl)
EcoRI	1µl
BamHI	1µl
10X buffer	2µl
Nuclease free water	Volume to make 20µl

Keep the restriction digestion at 37 °C for overnight. Gel purify the digested product and proceed for ligation.

Step6: Set up a ligation reaction and proceed for transformation

Digested sgRNA Cas9 vector	X µl (~50 ng)
Digested PCR product	1µl
10X T4 DNA ligase buffer	1µl
T4 DNA ligase	0.5 µl
Nuclease free water	Volume to make 10µl

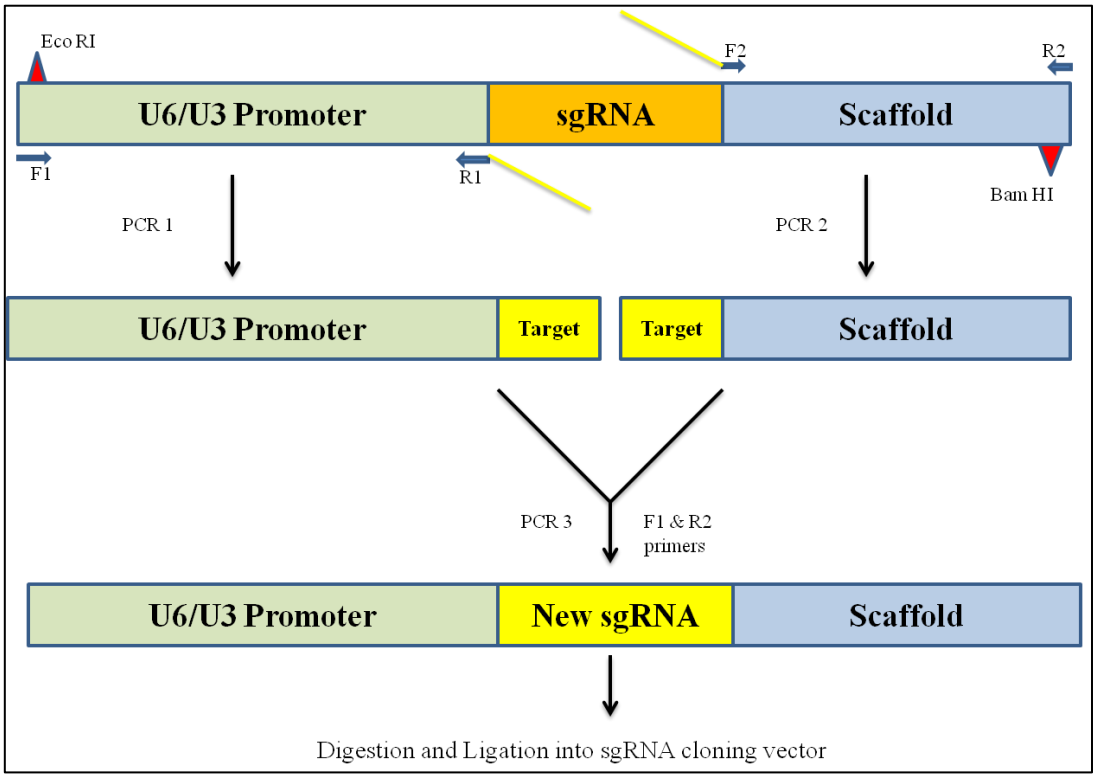

Figure 3. Diagrammatic representation of overlap extension PCR using different primer combinations.

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Chapter 5 Transformation of CRISPR/Cas9 vector in *E. coli.* and confirmation through restriction digestion

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Introduction and principle

Transformation is the process that allows a bacterial cell to take up the foreign DNA. To achieve bacterial transformation, the host bacterial cells should be made competent to uptake DNA. Calcium chloride has been used invariably to make bacterial cells competent as treatment of bacterial cell suspension with Calcium chloride makes cell membranes more permeable. This happens because Ca^{+2} ions attach to both negatively charged lipopolysaccharides of the membrane and DNA molecules. A heat shock of 42°C to competent bacterial cells further promotes the movement of DNA molecules from the surrounding medium into the host cells.

Materials required:

Conical flask, DNase, RNase-free tubes (0.2, 0.5 and 1.5, 5, 50 ml volume), *E. coli* DH5α strain, 37°C/ 42°C heat block, incubator shaker, Nuclease-free water, Centrifuge, Micro-centrifuge, Luria broth (LB), Luria agar (LA), $Cacl_2$ (0.1M), Kanamycin (50mg/ml), Vector, Insert, T4 DNA Ligase, Solution-I, Solution-II, Solution-III, Iso-propanol, 70% ethanol, Restriction enzymes, Agarose, Gel electrophoresis unit, gel documentation system

Protocol

I) Protocol for the transformation of CRISPR/Cas9 vector in *E. coli.* DH5α strain.

1. Protocol for competent cells preparation

Competent cells are prepared following the standard $CaCl_2$ method (Sambrook *et al.* 1989). *E. coli* strain DH5α (Nalidixic acid resistance) (Hanahan, 1983) is used for the preparation of competent cells for transformation. Such competent cells will be transformed using the CRISPR/Cas9 ligated vector.

A fresh 5 ml starter culture of *E. coli* is prepared from a single colony via overnight shaking at 200 rpm in an incubator shaker at 37°C. The next day, 1 ml inoculum of starter culture is added to 100 ml LB medium in a 250 ml conical flask containing antibiotic for selective growth and incubated at 37°C for 3-4 h with shaking at 200 rpm till bacterial O.D. reaches 0.5-0.6. Further growth of culture is stopped by incubating the culture flask on ice for 45-60 min. The culture is pelleted down by centrifuging at 5000 rpm for 10 min, suspended in 10 ml 0.1 M $CaCl_2$ and

further incubated for 30 min. Cells are again pelleted by centrifuging at 6000 rpm for 10 min and resuspended in fresh 1 ml of 0.1 M $CaCl_2$ containing 15% glycerol (a cryoprotectant). This suspension is kept on ice for 10 min and dispensed into 100 µl aliquots in sterile 1.5 ml centrifuge tubes. These tubes are snap chilled in liquid nitrogen and stored at -70°C for further usage.

2. Ligation reaction:

Ligation reaction is kept to ligate the two DNA fragments by forming a phosphodiester bond with the help of an enzyme called T4 DNA Ligase. The vector and insert ration are very important for successful ligation reaction. A calculator can be used to take the amount of vector and insert for ligation reaction at (https://nebiocalculator.neb.com/#!/ligation)

Reagents	**Volume**
Vector (20 ng)	X µl
Insert (60 ng)	X µl
T4 DNA Ligase buffer (10X)	2.0 µl
T4 DNA Ligase	1.0 µl
Nuclease free water	X µl
Total	20.0 µl

Gently mix the reaction by pipetting up and down and microfuge briefly. Incubate the tubes at 16°C or 22°C for 1h/overnight as per the recommendation by the manufacturer.

3. Transformation of *E. coli.* DH5α with ligation mix using the heat shock method

1. Take out competent cells from -70°C and allow it to thaw on ice for 5-10 min.
2. Add ligation mix (10 µl) to 100 µl competent cells and mix gently.
3. Incubate the mixture on the ice for 15-20 min.
4. Give a shock of 42°C for 90 sec and quickly transfer the tubes to the ice.
5. After 5 min, add 900 µl of LB and incubate at 37°C on a shaker at 200 rpm for 1 hour.
6. Harvest cells by centrifugation at 9,000 rpm for 3 min.
7. Discard the 900 µl supernatant and re-suspend the cells in rest 100 µl LB by pipetting.
8. Spread the suspension over the LA plate containing the kanamycin antibiotic (50 mg/l).
9. Incubate the plates overnight at 37°C for the growth of colonies.

II) Selection of transformants

The next day, transformed recombinant grew colonies (6 to 7) are selected for plasmid isolation.

Isolation of plasmid DNA and restriction digestion for the confirmation of cloning

Plasmid DNA Isolation principle

The alkaline lysis method (Brimboim and Doly, 1979) is a popular method in molecular biology protocols for the isolation of intact plasmids. The presence of detergent (sodium dodecyl sulphate, SDS) and alkaline (NaOH) condition helps in the lysis of bacterial cells and denaturation of the genomic and extra-chromosomal DNA molecules. Instant reversal of the alkaline conditions to acidic by adding Na-Acetate causes the rapid renaturation of the DNA molecules. Due to the smaller size of plasmid DNA, they renature very fast as compare to genomic DNA (larger size). Hence, they settle down in the pellet after centrifugation, and plasmid DNA is selectively isolated from the supernatant.

Protocol of plasmid DNA isolation

1. Pellet the overnight grew bacterial culture by centrifuging at ~12,000 rpm for 2-5 min.
2. Discard the supernatant and resuspend the pellet in 200 µl of **solution I**.
3. Add 200 µl of **solution II** (lysis buffer), mix gently by inverting the tubes till a clear lysate is formed.
4. Add 300 µl of **solution III** (neutralization buffer), mix by inverting the tubes for 2 min.
5. Centrifuge the tubes at 12,000 rpm for 5 min to remove clear supernatant from cell debris.
6. Transfer clear supernatant to a new tube and add a 2/3 volume of chilled isopropanol and mix it properly.
7. Centrifuge the tubes at 12,000 rpm for 10 min and decant the supernatant and wash the pellet with 500 µl 70% ethanol.
8. Air-dry the pellet at 37°C to remove residual ethanol and dissolve pelleted plasmid DNA in nuclease-free water.

Restriction digestion of CRISPR/Cas9 vector for confirmation of cloning

Now the isolated plasmid DNA is checked via restriction digestion for the presence of the gene of interest within the restriction sites. Restriction digestion reaction will be set up as follow.

Reagents	Volume
Plasmid DNA (1-2 µg)	X µl
Recommended Buffer (10X)	2.0 µl
Restriction enzyme I (10 U)	1.0 µl
Restriction enzyme II (10 U)	1.0 µl
Nuclease free water	X µl
Total	20.0 µl

A brief spin is to be given to settle the content of the tubes and incubate at 37°C for 1 hour.

Agarose gel electrophoresis to confirm the cloning

Electrophorese the Restricted plasmid at 1% agarose gel along with 1 kb marker to confirm the size of the fragment and vector. Document the gel in any gel documentation system.

Sequencing of CRISPR/Cas9 vector

Further, the CRISPR/Cas9 vector needs to be confirmed through sequencing for the presence of sgRNA.

Solutions for plasmid isolation

1. **Solution I**

   25 mM Tris. Cl (pH 8.0)

   10 mM EDTA (pH 8.0)

   50 mM Glucose

2. **Solution II**

   0.2 N NaOH

   1% SDS

3. **Solution III**

   Potassium acetate 3M

Bacterial Growth media

1. LB medium (pH 7.0)

Chemical	**g/l**
Yeast extract	5
Tryptone	10
NaCl	10

2. LA medium (pH 7.0)

Chemicals	**g/l**
Yeast extract	5
Tryptone	10
NaCl	10
Agar	15

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Chapter 6 Assembly of a multiplex CRISPR/Cas9 module in a binary vector

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Principle and Theory

When a gRNA-Cas9 module target a gene, then a double strand DNA break occur. In the plant system, due to DNA repair mechanism, these cut ends can join by two ways: i) non-homologous end joining (NHEJ), which resulted in indels and thus knock-out the gene function and ii) homology directed repair (HDR) of DNA if a template DNA strand is available, thus resulted in creation of new/altered DNA. One gRNA at a time target one site in the target gene and supposed to create knock-out mutant after non-homologous end joining. But some times it could be possible that the indel thus created is not stable and could be reverted back. Such condition frequently observed in case of plant viruses. Due to the ability of plant viruses to withstand mutation, many times NHEJ directed indels does not eliminate the plant virus rather they enable the virus to develop escape mutant which can overcome the effect of editing. Under such conditions where a single mutation in a target gene may not be very much effective to knockout the gene function, simultaneous targeting of multiple target sites of a gene is required using a multiplexed gRNA-Cas9 module. In the following chapter we will discuss about the development of such multiplexed gRNA-Cas9 module.

In this protocol we are following the methodology provided by Lowder *et al.*, 2005

Materials required:

i. Cloning vector: A vector toolkit comprising of 37 vectors for multiplexing of 1 to 8 gRNAs have been given in the paper. These vectors are classified into following groups:
   a. Golden Gate entry vector for individual gRNA cloning with either AtU6 or AtU3 promoters (e.g. pYPQ131A/B, pYPQ132A/B, pYPQ133A/B, pYPQ141A/B etc)
   b. Golden Gate multiplex gRNA recipient and gateway entry vectors with combinations of BsaI sites and gateway compatible sites (e.g. pYPQ142, pYPQ143 etc)
   c. Gateway compatible Cas9 expression vector with 35S promoter (e.g. pYPQ150)
   d. Gateway recipient binary vector (e.g. pEarley Gate 100)

ii. Restriction enzymes:
   a. BglII
   b. SalI-HF
   c. EPS3I
   d. BsaI

iii. Other enzymes and reagents:
   a. T4 ligase
   b. T4 Polynucleotide Kinase
   c. LR Clonase II
   d. DTT

iv. Bacterial competent cell:
   a. *E. coli* DB 3.1 cell for maintenance of plasmids
   b. *E. coli* DH5□□cell for cloning

v. Antibiotic:
   a. Tetracycline (5ng/ul)
   b. Spectinomycin (100 µg/ml)
   c. Kanamycin (50 µg/ml)

Detailed Protocol:

The entire protocol was performed in three steps:

Step1. Cloning individual guide RNA (gRNA) into expression vectors
Step2. Golden Gate Assembly of 2 or 3 guide RNAs
Step 3. Gateway Assembly of Multiplex CRISPR-Cas9 system into a binary vector

The details of these steps are described below. Entire protocol is summarized as a flow chart.

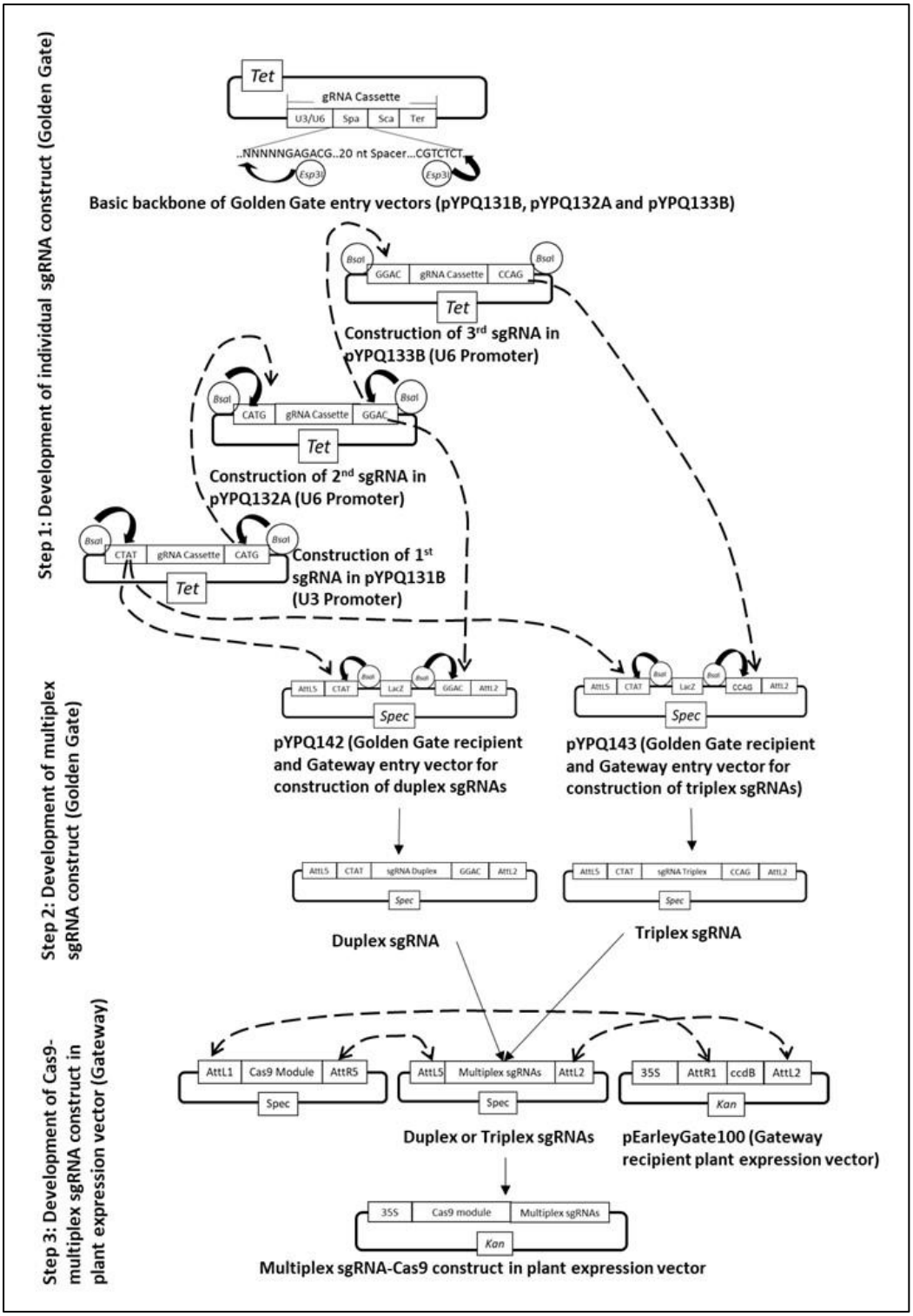
Step1. Cloning individual guide RNA (gRNA) into expression vectors

Suppose we take two vectors pYPQ131B (AtU3 promoter) and pYPQ132A (AtU6 promoter) and we will clone two gRNAs into them individually.

I. Linearize guide RNA expression plasmids

1. First digestion with BglII and SalI (Optional but recommended for zero-background cloning)

Component	Amount (µl)
H_2O	14
gRNA plasmid (~100 ng/µl)	20
10X NEB buffer 3.1	4
BglII (10 u/µl; NEB)	1
SalI-HF (10 u/ µl; NEB)	1



Total	40

Incubate at 37^0C for 3 hrs.

2. Second digestion with Esp3I (BsmBI)

Purify 1st digestion products using Qiagen PCR purification kit, elute DNA with 35 µl ddH_2O, set up digestion reaction as follows:

Component	Amount (µl)
Digested gRNA plasmid (from step 1)	12
10X OPTIZYME buffer 4	4
DTT (20 mM)	2
EPS3I (10 u/µl; Thermo Scientific)	2
Total	40

Incubate at 37 ^{0}C, O/N

Inactivate enzymes at 80^{0}C denature for 20 min, purify the vector using Qiagen PCR purification kit and quantify DNA concentration using Nanodrop.

II. Cloning Oligos into linearized pYPQ131 vector

The protospacer sequence (20 nt preceeding to PAM) will be synthesized as primer oligo in both forward and reverse orientation. Remember PAM sequence is not a part of sgRNA

3. Oligo phosphorylation and annealing

Component	Amount (µl)
sgRNA oligo forward (100 µM)	1
sgRNA oligo reverse (100 µM)	1
10X T4 Polynucleotide Kinase buffer	1
T4 Polynucleotide Kinase (10 u/µl; NEB)	0.5
ddH_2O	6.5
Total	10

Phosphorylate and anneal the oligos using 37 ^{0}C for 30 min; 95 ^{o}C for 5 min; ramp down to 25 ^{o}C at 5 ^{o}C min-1 (i.e., 0.08 ^{o}C/second) using a thermocycler (alternatively: cool down in boiled water).

4. Ligate oligos into linearized gRNA expression vector and transformation of E.coli DH5α cells

Component	Amount (µl)
Linearized gRNA plasmid	1
Diluted annealed Oligos (1:200 dilution)	1
10X NEB T4 ligase buffer	1
T4 ligase	0.5
ddH_2O	6.5
Total	10

Incubate at room temperature for 1 hr.

5. Transform E.coli DH5α cells and plate transformed cells on a Tet+ (5ng/ul) LB plate; 37 ^{0}C, O/N

6. Mini-prep two independent clones and verify gRNAs by Sanger sequencing with primer pTC14-F2 (for pYPQ131, 132 and 133) or M13-F (for pYPQ141).

Step2. Golden Gate Assembly of 2 or 3 guide RNAs

1. Set up Golden Gate reaction as following:

Assembly of 2 guide RNAs

Component	Amount (µl)
H_2O	5
10X T4 DNA ligase buffer (NEB)	1
pYPQ142 (100 ng/ µl)	1
pYPQ131-gRNA1 (100 ng/ µl)	1
pYPQ132-gRNA2 (100 ng/ µl)	1
BsaI (NEB)	0.5
T4 DNA ligase (NEB)	0.5
Total	10

Assembly of 3 guide RNAs

Component	Amount (µl)
H_2O	4
10X T4 DNA ligase buffer (NEB)	1
pYPQ142 (100 ng/ µl)	1
pYPQ131-gRNA1 (100 ng/ µl)	1
pYPQ132-gRNA2 (100 ng/ µl)	1
pYPQ133-gRNA3 (100 ng/ µl)	1
BsaI (NEB)	0.5
T4 DNA ligase (NEB)	0.5
Total	10

2. Run Golden Gate program in a thermocycler as follows:

37 ^{0}C, 5 min
16 ^{0}C, 10 min } 10 cycles
50 ^{0}C, 5 min
80 ^{0}C, 5 min
Hold at 10 ^{0}C

3. Transform E. coli DH5α cells and plate transformed cells on a Spe$^+$ (100 µg/ml) LBplate. [Optional: Blue-white screen can be applied because guide RNA expression cassettes will replace the LacZ gene in pYPQ142 or pYPQ143 recipient plasmid]

4. Mini-prep two independent clones and verify by restriction digestion

Step 3. Gateway Assembly of Multiplex CRISPR-Cas9 system into a binary vector

1. Set up Gateway LR reaction as following:

Component	Amount (µl)
Cas9 entry vector (pYPQ150) (25ng/ µl)	2
Multiplex Guide RNA entry vector (pYPQ142/143) (25ng/ µl)	2
Destination vector (pEarleyGate) (100 ng/ µl)	2
LR Clonase II	1
Total	7

Incubate at room temperature for O/N

2. Transform E. coli DH5α cells and plate transformed cells on a Kan^+ (50 µg/ml) LB plate
3. Mini-prep two independent clones and verify by restriction digestion

Reference

Lowder LG *et al.* 2005. A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. Plant Physiol. 169: 971-985.

Chapter 7 *In planta* transient delivery CRISPR/Cas9 module through Agroinoculation

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Principle Theory

Agrobacterium tumefaciens is a gram-negative plant pathogenic bacterium causing gall or tumor-like growths on infected plants. These tumors are driven by the movement of transfer DNA (T-DNA) from the bacterial Ti-plasmid to the genome of the eukaryotic host. Agrobacterium tumefaciens-mediated such horizontal gene transfer system is a useful technique for the insertion of modified DNA into cells to create genetically modified plant lines if co-cultured with callus of the plant. In adult plant with fully differentiated tissue, agrobacterium based binary vectors with left and right border sequences can deliver the gene of interest but under such condition the gene of interest does not integrate with chromosomal DNA rather expressed in a transient manner. Transient assay of expression of a protein many times preferred over transgenic assay due to its quick mode of assay to select the superior gene construct. Before developing a transgenic plant using CRISPR/Cas9 module it is essential to check their *in planta* efficacy under a transient assay. We can quickly assay the effectiveness of different CRISPR/Cas9 construct against plant viruses using such transient method.

For transient assay the agrobacterium cells were made competent to uptake the CRISPR/Cas9 module. Electrocompetent cells are much more efficient than the chemically competent cells. The electrocompetent cell is used in the electroporation method. Electroporation is the most versatile and efficient transformation technique, which employs accurately pulsed electric currents to induce transient gaps in the phospholipid bilayer of cells. Then the DNA can easily be taken up by the agrobacterium. The agro-culture with the CRISPR/Cas9 construct was then infiltrated in the *Nicotiana benthamiana* leaf. For assaying the effectiveness of the CRISPR/Cas9 constructs, after inoculation of CRISPR/Cas9 modules, virus culture/infectious clone is inoculated into the same leaf but in different location. Generally CRISPR/Cas9 modules are inoculated near to the petiole portion of the leaf while virus culture is inoculated at the apex, so that after replication when the viral genome come in contact with CRISPR/Cas9 system it will get cleaved.

Materials required:

1. Disarmed A. tumefaciens Strain (such as GV3101)

2. CRISPR/Cas9 construct in binary vector (here we will use pEarley Gate 100)
3. Luria Broth (LB), Luria Agar (LA) and SOC medium
4. Autoclaved 10% Glycerol
5. 0.2 µm filter
6. Antibiotic – a. Kanamycin (50 µg/ml), Rifampicin (50 µg/ml)
7. Electroporator

The entire process has three steps: agrobacterium electro competent cell preparation, electroporation of agrobacterium and Agroinoculation.

I. Agrobacterium electro competent cell preparation

- Streak Agrobacterium GV3101 cells from a plate or a stab onto a LA plate (with Rif+ antibiotic selection) and incubate at 28°C for 36-48 hours to allow to grow.
- Use a well-isolated colony to inoculate 50 ml of LB in a 250 ml flask and grow cells with vigorous aeration at 28°C for O/N. Take 1 ml of the culture and inoculate freshly in 50 ml LB with Rif selection and grow until the OD_{600} nm reaches to 0.5-1.
- When cells are ready to harvest chill screw cap tubes (50 ml) on ice for 15 -30 minutes. Pour the bacterial culture into chilled screw cap tubes (50 ml).
- Pelletize the cells by centrifuging at 4000 g for 15 minutes at 4°C
- Remove supernatant and resuspend the pellet in each tube with 10 ml ice cold 10% glycerol
- Pelletize the cells by centrifuging at 4000 g for 15 minutes at 4°C
- Remove supernatant and resuspend the pellet in each tube with 10 ml ice cold 10% glycerol
- Centrifuge at 4000 g in bottles for 15 minutes at 4°C
- Remove supernatant and resuspend the pellet in 2 -3 ml ice cold 10% glycerol.
- Store in 1.5 ml microfuge tubes in 50 µl aliquots, freeze in liquid nitrogen, store in -80°C, cells are good for at last 6 months.

II. Electroporation of Agrobacterium

- Pipette the DNA samples (CRISPR/Cas9 plasmid) up to 5 µl to be electroporated into sterile chilled 1.5 ml microfugetubes. The DNA should be in either water or TE. Place tubes on ice.
- Thaw the electrocompetent *A. tumefaciens* cells on ice. For each DNA sample to be electroporated, add 20-50 µl of electrocompetent cells to each DNA sample; gently tap the tubes to mix and keep in ice for 10 min.
- Transfer the DNA-cell samples to the chilled electroporation cuvettes (need to be chilled before using) and tap the suspension to the bottom of the tube. Place the cuvette in the electroporator chamber slide. Push the slide into the electroporator chamber until

the cuvette is seated between the contacts in the base of the chamber. Pulse once at1.4 to 2.2 KV for 5 milliseconds until the beep.

- Remove the cuvette from the chamber and immediately add 1 mL SOC medium to cuvette, slowly mix by pipette up and down and transfer the entire content to a microfuge tube
- Incubate at 28 ^{0}C (cells will die at 37C) for 2-3 hours before plating ~200 µl on LA plates with Kan and Rif selection.
- Incubate plates for 48 hrs at 28 °C. After 48 hrs pick few colonies and prepare a master plate with appropriate antibiotic selection. Confirm colonies by colony PCR.

III. Agroculture preparation and Agroinoculation

- Select individual agro colony and grow in 2 mL LB under antibiotic selection on a shaker at 28°C with 250 rpm for O/N.
- Take 500 ul of O/N grown culture and add 10 ml fresh LB medium with antibiotic. Grow 28°C with 250 rpm for 2 hrs.
- Harvest the bacteria by centrifugation at 4 ^{0}C for 10 min at 4000 g, resuspend the bacterial pellets in infiltration medium (10 m*M* MES pH 5.6, 10 m*M* $MgCl_2$) until the OD_{600} reach to 0.5 and then incubate at room temperature with gentle shaking for 1-2 hrs.
- Add 150 µM acetosyringone and incubate at room temperature for at least 2 h to allow the induction of *A. tumefaciens* virulence (*vir*) genes. Acetosyringone is light sensitive so cover the tubes with aluminium foil.
- Use a 1.0- to 2.5-mL syringe without needle to infiltrate fully expanded leaves of 4- to 5-week-old *N. benthamiana* plants. Use a needle to create a small incision/injury into the abaxial side of the plant leaf. Then, press the syringe tip to the incision and inject the bacterial solution into the leaf airspace with gentle pressure.

Agroinfiltration

Buffer and chemical preparation

1. Preparation of 0.5 M MES buffer pH5.6 stock for 1 L

- Suspend 97.62 g of MES free acid (mw = 195.24 g/mol) in 750 mL of ddH_2O.
- Adjust to desired pH using 10N NaOH.
- Fill to final volume of 1 L with ddH2O.
- Filter sterilize(recommended) or autoclave.
- Store at 4°C

2. Preparation of 1 M $MgCl_2$ stock solution

Dissolve 203.3 g of $MgCl_2$, 6H2O in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

3. Preparation of acetosyringone 100mM Stock

Dissolve 0.3924 g in 12 ml 95% ethanol, then add 8 ml of sterile milli-Q water to equal 20 ml. Filter Sterilize and store at -20°C in amber bottle as acetosyringone is light sensative.

Reference

Weigel D amd Glazebrook J. 2006. Transformation of Agrobacterium using electroporation. Cold Spring Harb Protoc. doi: 10.1101/pdb.prot4665.

Chapter 8

Real time PCR for detection of viral load after editing of the viral genome

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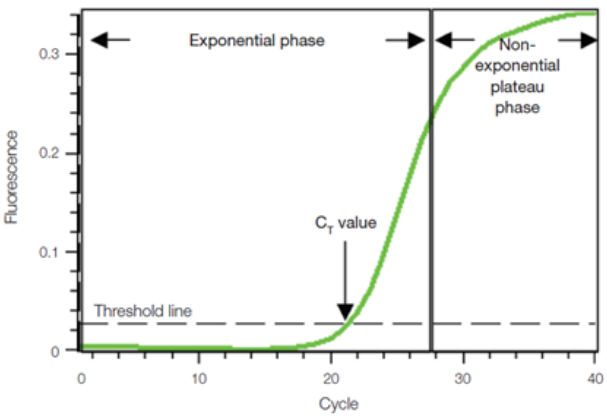
Principle and theory

Real time PCR is an improved PCR technique in which the progress of a PCR reaction or accumulation of amplified product is monitored in real time. In contrast, conventional PCR detect the amplified product, or amplicon by running DNA on an agarose gel after the reaction has finished. So, conventional PCR detect amplicon by an end-point analysis. In real-time PCR we use fluorescent molecule (DNA-binding dyes and fluorescently labeled sequence-specific primers or probes) that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescence amplification signal is detected by special fluorescence detection modules in a specialized thermal cycler. The measured fluorescence reflects the amount of amplified product in each cycle.

There are several advantages of real-time PCR over the conventional PCR:

i. Real-time PCR allows us to quantify the DNA so it is also called qPCR.
ii. We can determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range.
iii. As result is obtained during the PCR cycle post analysis is not required so time is reduced
iv. No chance of contamination



Courtesy: Real-time PCR application Guide : Bio Rad

Any PCR reaction, occurs through two distinct phases, an exponential phase followed by a non-exponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase. In conventional PCR as we detect the end product, we can not able to estimate quantity of the PCR product.

In real-time PCR, after certain cycles when enough amplified product accumulates it yield a detectable fluorescent signal. The cycle number at which this detection of fluorescence occurs is called the threshold cycle, or C_T. The C_T of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. So, C_T value and initial DNA

template concentration are inversely proportional. More initial DNA indicates a low C_T value as it reaches the detectable limit in early cycle. This relationship forms the basis for the quantitative aspect of real-time PCR.

The variety of fluorescent chemistries, which form basis of real-time PCR, can be grouped into two major types: 1) DNA-binding dyes (SYBR Green I), and 2) dye-labeled, sequence-specific oligonucleotide primers or probes (molecular beacons and TaqMan, hybridization, and Eclipse probes, and Amplifluor, Scorpions, LUX, and BD QZyme primers). The most commonly used chemistries for real-time PCR are the DNA-binding SYBR Green I dye and TaqMan hydrolysis probe. DNA-binding dyes are most preferable because these assays are easier to design, are faster to set up, and are more cost-effective. The most commonly used DNA-binding dye for real-time PCR is SYBR Green I, which binds non-specifically to any double-stranded DNA (dsDNA). When in a solution free SYBR Green I exhibits little fluorescence, but upon binding with dsDNA its fluorescence increases up to 1,000-fold. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified.

The real time PCR primers are designed with high GC content, Tm between 50ºC and 65ºC and with less secondary structure, no overlap between primers to avoid primer-dimer formation. Typically the amplicon size of a real-time PCR should be 75–200 bp.

Through real-time PCR we can quantify the data in two ways: absolute quantification and relative quantification. In absolute quantification we measure the quantity of the test samples by comparing the C_T values of the test samples to a standard curve. In this way we can measure the quantity of nucleic acid (copy number, µg) per given amount of sample (e.g. per cell, per µg of total RNA). Relative quantification is achieved through a fold analysis of the relative amount (fold difference) of a target nucleic acid of test and control sample. As different biological samples are analyzed and compared simultaneously, a qPCR experiment must be normalized so that the data become biologically meaningful. The normalizer can be the number of cells used for template preparation, µg of nucleic acid used as PCR template, or the expression level of a reference gene. The first two are more commonly used for absolute quantification, whereas the third is typically used for relative quantification. A reference gene is one whose expression level is constant across all test samples and whose expression is not affected by the experimental treatment under study. The use of a reference gene is advantageous in cases where the precise quantification of input RNA amount is not possible, such as in cases where only a small amount of starting template is available.

When comparing multiple samples using relative quantification, one of the samples is usually chosen as the calibrator, and the expression of the target gene in all other samples is expressed as an increase or decrease relative to the calibrator. To determine the relative expression of a target gene in the test sample and calibrator sample using reference gene(s) as the normalizer, the expression levels of both the target and the reference genes need to be determined. So, C_T value of the target gene and reference gene with respect to both test and calibrator samples need to be determined.

After the C_T values are measured, different methods can be used to determine the expression level of the target gene in the test sample relative to the calibrator sample. In the following

sections, we present three methods for relative quantification using a reference gene: 1) the Livak method, also known as the $2^{-\Delta\Delta C_T}$ method, 2) the ΔC_T method using a reference gene, and 3) the Pfaffl method.

The $2^{-\Delta\Delta C_T}$ method for relative gene expression analysis is widely used and easy to perform. This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other. Before using the $2^{-\Delta\Delta C_T}$ method, it is essential to verify the assumptions by determining the amplification efficiencies of the target and the reference gene. Once you have established that the target and reference genes have similar and nearly 100% amplification efficiencies, you can determine the relative difference in expression level of your target gene in different samples using the steps below:

First, normalize the C_T of the target gene to that of the reference (ref) gene, for both the test sample and the calibrator sample:

$$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(ref,\ test)}$$

$$\Delta C_{T(calibrator)} = C_{T(target,\ calibrator)} - C_{T(ref,\ calibrator)}$$

Second, normalize the ΔC_T of the test sample to the ΔC_T of the calibrator:

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

Finally, calculate the expression ratio:

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

The result obtained is the fold increase (or decrease) of the target gene in the test sample relative to the calibrator sample and is normalized to the expression of a reference gene. Normalizing the expression of the target gene to that of the reference gene compensates for any difference in the amount of sample tissue.

Materials required:

- DNA or RNA isolated from plant
- SYBR Green supermix, BioRad, USA
- Forward and Reverse primers
- Real-time PCR machine

Detailed protocol:

1. Take 100 ng of template DNA/RNA/cDNA. Take test sample, calibrator sample and a reference gene.
2. **SYBR Green real-time PCR:** Prepare 25µl reaction mixtures containing 1x Master Mix (iQTM SYBR Green supermix, BioRad, USA) buffer and 800 nM of each primer.
3. Place the PCR tubes in the iQ5 machine (Bio-Rad, USA) and carry out the PCR using following cycling condition:

Enzyme activation	95˚C for 5 min	
Denaturation	95˚C for 15 sec	40 cycles
Annealing	60˚C* for 20 sec	
Extension	72˚C for 30 sec	

*depends upon Tm of primers

Result:

50 ng DNAs isolated from both gRNA-Cas9 treated-virus inoculated (test) and gRNA-Cas9 untreated-virus inoculated (calibrator) samples. We want to measure the relative quantification of viral load in these two samples to understand whether gRNA-Cas9 treatment could reduce the viral load. For viral load measurement we chose a fragment of viral Rep gene (target gene) and Actin gene of host is used as a reference gene as its expression does not vary between test and calibrator samples. The C_T values for each sample are shown below:

Sample	C_T Rep gene (target)	C_T Actin (reference)
gRNA-Cas9 treated virus inoculated (Test)	19.21	26.27
gRNA-Cas9 untreated virus inoculated (Calibrator)	18.53	26.30

First C_T of the target gene is normalized to the C_T of the reference gene for both the test sample and the calibrator sample by calculating ΔC_T

$$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(ref,\ test)} = 19.21\text{-}26.27 = -7.06$$

$$\Delta C_{T(calibrator)} = C_{T(target,\ calibrator)} - C_{T(ref,\ calibrator)} = 18.53\text{-}26.30 = -7.77$$

Second, normalize the ΔC_T of the test sample to the ΔC_T of the calibrator:

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)} = -7.06\text{-}(-7.77) = 0.71$$

Finally, calculate the expression ratio:

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio} = 0.611320139$$

In the test samples virus accumulation is 0.61 fold than the calibrator where no gRNA-Cas9 was treated. So in the gRNA-Cas9 treated plant virus accumulation is lowered.

Reference

Bio-Rad Application Guide: Web Resource:

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf

Chapter 9 Isolation and transformation of Protoplast

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Principle:

Protoplasts are isolated by treating tissues with a mixture of cell wall degrading enzyme in solution, which contains an osmotic stabilizer. A most suitable source of protoplasts is mesophyll tissue from fully expanded leaves of young plants or new shoots. The release of protoplast is very much dependent on the nature and composition of enzymes used to digest the cell wall. There are three primary components of the cell wall which have been identified as cellulose, hemicellulose and pectin substance. Pectinase (macerozyme) mainly degrades the middle lamella while cellulase and hemicellulase degrade the cellulose and hemicellulosic components of the cell wall. During this enzymatic treatment, the protoplast obtained should be stabilized because the mechanical barrier of the cell wall which offered support has been broken. For this reason, an osmoticum is added which prevents the protoplast from bursting.

Materials required:

- Young leaves from 4-week old plants
- Cellulase R10
- MacerozymeR10
- PEG-4000
- 70µ Cell strainer
- 50 mm Petri dishes
- 50 ml falcon tubes (conical)
- Scalpel
- Chemicals: $CaCl_2$, $MgCl_2$, KCl, NaCl
- MES
- Mannitol
- Equipment: Rocker shaker, refrigerated centrifuge, hemocytometer, microscope

Detailed protocol:

1. Prepare the enzyme solution fresh or thaw it if stored at -20°C.
2. Use 4-week old plants grown at 20-22°C in a short day (10-12 h light).
3. Harvest largest young leaves (3-4 per plant) and remove midrib, top and bottom part. (2-4 plants should yield ~4-8 x 10^6 protoplasts)
4. Slice the rest on clean paper into ~1mm strips.
5. Transfer the leaf material to a ~6 ml enzyme solution into 55 mm Petri dishes.
6. Vacuum infiltration: apply vacuum until it reaches 0.8 bar for 5 minutes (twice).
7. Incubate at 25°C for 2.5-3h in total.
8. Prepare a PEG solution, at least 1 h before use.

9. Work on ice afterward, use cut tips to pipette protoplasts.
10. Filter through a 70µ nylon cell strainer into a 50 ml falcon tube and rinse with the same volume of cold buffer W5.
11. Centrifuge protoplasts at 100g for 2 min at 4°C.
12. Protoplasts should be seen as a green pellet at the bottom of the tube. Remove most of the supernatant.
13. Resuspend protoplasts in the same volume of cold buffer W5.
14. Once again spin the protoplasts for 1-2 min at 100g and 4°C. Remove most of the supernatant.
15. Observe the dissolved protoplasts in W5 under the microscope and quantify by using the hemocytometer.
16. Dilute the quantified protoplasts to the desired concentration in MMG (start with a smaller volume).
17. Optional: stain an aliquot with FDA for counting vital protoplasts. For this 100 µl protoplasts added with 0.5 µl FDA (5 mg/ml stock in acetone) and keep in dark for 5-10 min at room temperature. Observe the PPs under the microscope.
18. Count protoplasts using a Fuchs Rosenthal hemocytometer, use cut pipette tips. To count cells using a hemocytometer, count 4 large fields (two large fields on each side of the hemocytometer, 64 small fields in total). Add 15-20µl of cell suspension between the hemocytometer and cover glass. The goal is to have roughly 100-200 cells/square. Count the number of cells in all four outer squares divide by four (the mean number of cells/square).

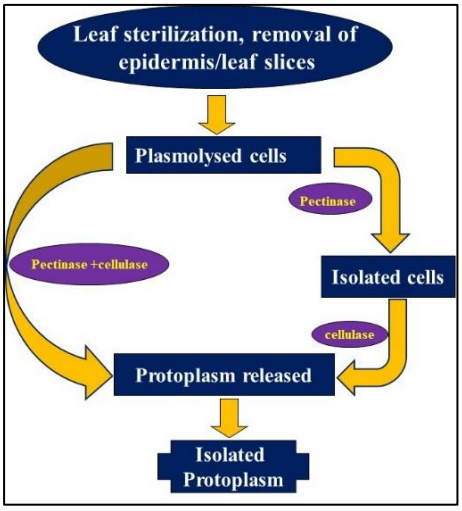
Protoplast transformation:

1. Resuspend PPs in MMG at 2 x 10^5 (single transformation) or 4 x 10^5 (double transformation) protoplasts/ml
2. Keep on ice until use.
3. Handle 6 samples (maximum) each time.
4. Put the DNA in a 2 ml round bottom reaction tube and add the protoplasts. Mix by flicking the tube.
5. Add PEG solution and mix by flicking the tube until the solution becomes homogenous

Single transformation			
Components/PPs	20,000 PPs	40,000 PPs	60,000 PPs
Plasmid DNA	4 µg	8 µg	12 µg
PPs solution (2x10^5/ml)	100 µl	200 µl	300 µl
PEG solution	100 µl + Volume of plasmid	200 µl + Volume of plasmid	300 µl + Volume of plasmid
Double transformation			
Components/PPs	40,000 PPs	80,000 PPs	120,000 PPs
Plasmid DNA-1	4 µg	8 µg	12 µg
Plasmid DNA-2	4 µg	8 µg	12 µg
PPs solution (2x10^5/ml)	100 µl	200 µl	300 µl
PEG solution	100 µl + Volume of plasmid	200 µl + Volume of plasmid	300 µl + Volume of plasmid

6. Incubate for 20-30 min at RT. Stay at RT now.
7. Add 500 µl of buffer W5 to stop the transformation.
8. Centrifuge for 1-2 min at 100g.
9. Take out most of the supernatant
10. Add 500µl of buffer WI
11. Incubate solution in open tubes covered with Nesco film (allows gas exchange) in the growth chamber (16-18 h)

Illustration



Results and conclusion

The protoplasts isolated by enzymatic method could be viewed and quantified under the microscope. The healthy protoplast will be spherical whereas dead one will be shrunken or broken.

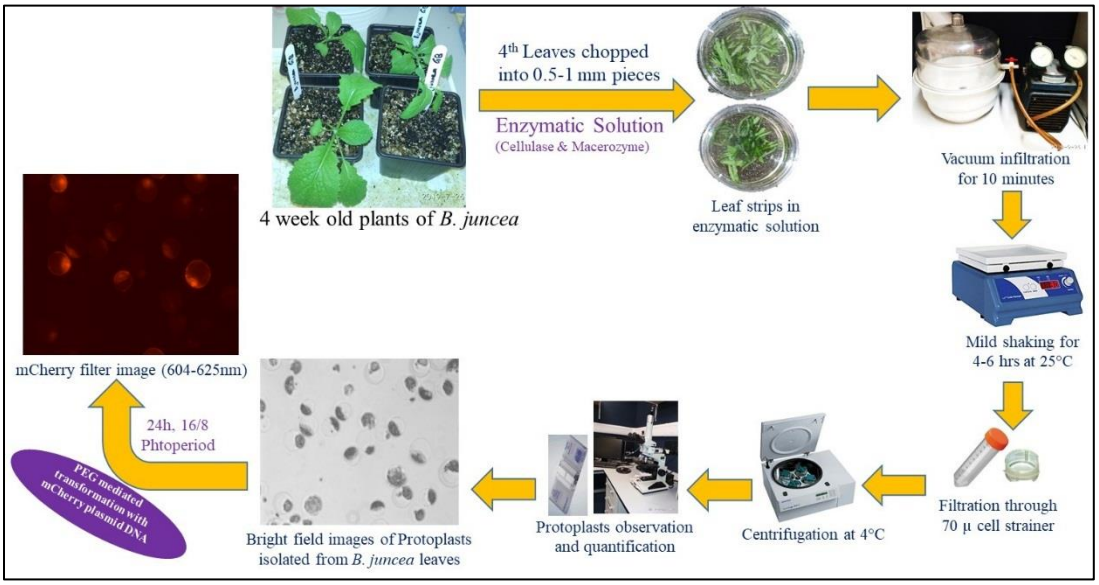


Figure 1: A schematic representation of various steps

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Chemical preparation & buffer compositions

- 0.2 M MES 4-(N- Morpholino) ethane sulfonic acid), pH5.7. Sterilize using a 0.45µm membrane filter.
- 0.8 M Mannitol, sterilize by filtration.
- 1M $CaCl_2$, sterilize by autoclaving.
- 1M KCl, sterilize by autoclaving.
- 1M $MgCl_2$, sterilize by autoclaving.
- 3M NaCl, sterilize by autoclaving.

1. Enzyme solution (ES)

Add the following component sequentially into a sterile 50 ml falcon tube

S.No.	Components	Stock solution	For 20 ml	For 40 ml
i.	H_2O	-	7	14
ii.	20 mM MES, Ph5.7	0.2 M	2	4
iii.	0.4 M Mannitol	0.8M	10	20
iv.	20 mM KCl	1.0 M	0.4	0.8
v.	1.5% (w/v) cellulase R-10	-	0.3g	0.6 g
vi.	0.4% (w/v) Macerozyme R-10	-	0.08g	0.16 g
Prepare fresh and heat up to 42°C for 10 minutes to aid enzyme solubility				
vii.	10 mM $CaCl_2$	1.0 M	0.2	0.4
viii.	0.1% BSA	10%	0.2	0.4

Filter the final enzyme solution through a 0.2 µm membrane filter. Excess can be stored at -20°C.

2. PEG Solution

S.No.	Components	Different volumes to make				
i.	PEG-4000	1g	2g	4g	6g	8g
ii.	0.8M Mannitol	0.625 ml	1.25 ml	2.5 ml	3.75 ml	5.0 ml
iii.	1M $CaCl_2$	0.25 ml	0.5 ml	1.0 ml	1.5 ml	2.0 ml
iv.	H_2O	0.75 ml	1.5 ml	3.0 ml	4.5 ml	6.0 ml
	Total Volume	**2.5 ml**	**5.0 ml**	**10 ml**	**15 ml**	**20 ml**

3. Washing and Incubation (WI) Solution

S. No.	Components	Stock solution	For 100 ml
i.	4 mM MES, pH 5.7	0.2M	2 ml
ii.	0.5 M Mannitol	0.8M	62.5 ml
iii.	20 mM KCL	1M	2 ml
iv.	H_2O	-	33.5 ml

- Autoclave and store in the fridge.

4. Washing Solution 5 (W5)

S. No.	Components	Stock solution	For 100 ml
i.	2 mM MES, pH 5.7	0.2M	1 ml
ii.	154 mM NaCl	3M	3.08 ml
iii.	125 mM $CaCl_2$	1M	12.5 ml
iv.	5 mM KCL	1M	0.5 ml
iv.	H_2O	-	82.92 ml

- Autoclave and store in the fridge.

5. MMG Solution

S. No.	Components	Stock solution	For 100 ml
i.	4 mM MES, pH 5.7	0.2M	2 ml
ii.	0.4 M Mannitol	0.8M	50 ml
iii.	15 mM $MgCl_2$	1M	1.5 ml
iv.	H_2O	-	46.5 ml

- Autoclave and store in the fridge.

Abbreviations

MES: 4-(N-Morpholino) ethane sulfonic acid

PPS: Protoplasts

W5: Washing solution

WI: Washing and Incubation Solution

MMG: Mannitol, MES and $MgCl_2$ solution

PEG: Polyethylene glycol

FDA: Fluorescein diacetate

Chapter 10 Agrobacterium mediated transformation of CRISPR/Cas9 vector

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Introduction

Agrobacterium tumefaciens is a gram-negative rod-shaped bacterium (Jin, S.) and plant pathogen that causes tumors like growths (crown gall disease) on infected plants. The bacterium contains a plasmid (the tumor–inducing or Ti plasmid), part of which (the T-DNA) integrates into the host plant chromosomes (**Figure 1**). This horizontal gene transfer system, called *Agrobacterium tumefaciens*-mediated transformation (AtMT), has become a useful technique for the insertion of modified DNA into cells to create genetically modified plant lines. The initial introduction of plasmid into *Agrobacterium* can be achieved through various techniques, including freeze-thaw method (Weigel and Glazebrook, 2006). The Agrobacterium strain harbouring the plant transformation construct is allowed to infect the plant tissues (explant). The transgenic plant is recovered from the infected plant tissues through a regime of plant tissue culture which ensures regeneration of new shoots/plants from the cells in which T-DNA transfer has occurred.

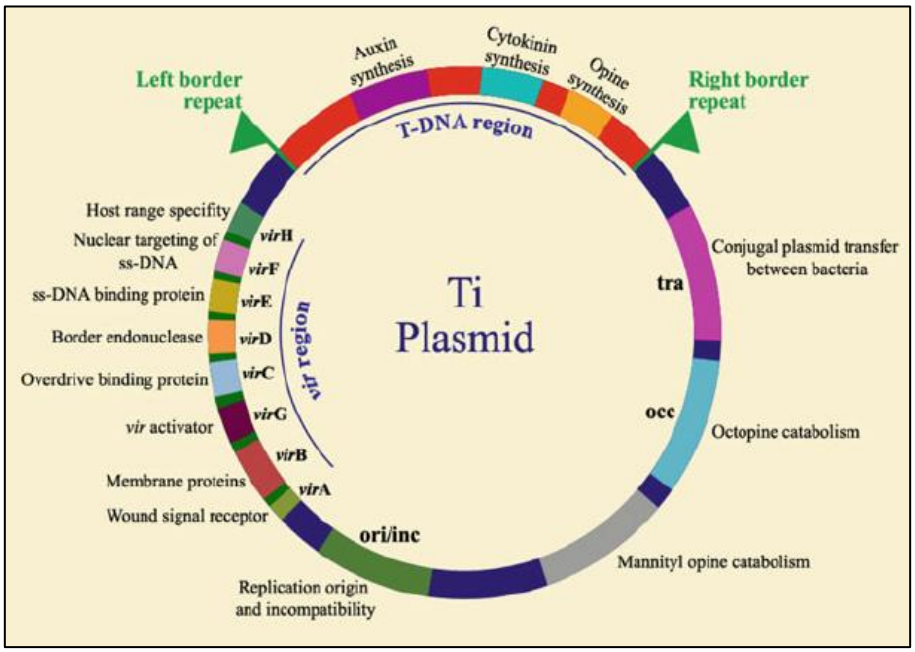

Figure 1: Schematic representation of a Ti plasmid

Materials Required

1. ***Agrobacterium tumefaciens* Growth Media**

Luria Broth Medium (pH 7.0)

Chemical	Amount
Tryptone	10 gm/L
Yeast Extract	5 gm/L
NaCl	10 gm/L

Luria Agar Medium (pH 7.0)

Chemical	Amount
Tryptone	10 gm/L
Yeast Extract	5 gm/L
NaCl	10 gm/L
Agar	15 gm/L

SOC Medium (pH 7.0)

Chemical	Amount
Tryptone	20 gm/L
Yeast Extract	5 gm/L
NaCl	10 gm/L
MgSO4	0.96gm/L
Glucose	3.6 gm/L
KCl	0.186 gm/L

2. Recombinant plasmid 10 gm/L

3.100mM $MgCl_2$

4 .100mM $CaCl_2$

5. Autoclaved 1.5ml eppendorf tubes

6. 50ml falcon tubes

7. Glycerol
8. Ice
9. Nuclease free water
10. Liqiud Nitrogen
11. Culture vials and conical flask

Antibiotics stock solution

Rifampicin (25mg/ml)

Weigh 0.25 g of rifampicin. Add 10 ml of 100% methanol. Dissolve completely. Stock may be kept at -20°C for 1 year. Rifampicin may precipitate in the freezer. Dissolve again before use

Gentamycin (50mg/ml)

Weigh 0.5 g of Gentamicin. Add 10 ml of sterile H2O. Dissolve completely. Prewet a 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water. Sterilize Gentamicin Stock through the prepared 0.22 µm syringe filter. Stock may be kept at -20°C for 1 year.

Kanamycin (50mg/ml)

Weigh 0.5 g of Kanamycin. Add 10 ml of sterile H2O. Dissolve completely. Prewet a 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water. Sterilize Kanamycin Stock through the prepared 0.22 µm syringe filter. Stock may be kept at -20°C for 1 year.

Instruments:

1. Spectrophotometer
2. Centrifuge (4 °C)
3. Incubator and shaker
4. pH meter

Detailed Protocol-

I) Preparation of competent cells of *A. tumefaciens* strain GV3101:

1. Streak *A. tumefaciens* strain GV3101 from a glycerol stock onto a LB plate (Gentamicin-50mg/ml and Rifampicin -25mg/ml) and incubate at 28°C for 36-48 hours to allow to grow.
2. Inoculate a single colony of *A. tumefaciens* strain GV3101 from LA plate in 10 ml SOC broth with gentamicin (50mg/ml) and rifampicin (25mg/ml) and incubate at 28°C with continuous shaking at 200rpm for 48 h.

3. Inoculate 3 ml of the overnight grown culture in 100 ml SOC broth in 500 ml conical flask and incubate at 28°C with continuous shaking at 200 rpm for 5-6 h until the $O.D_{550}$ reaches ~0.5-0.8.
4. When the OD550 reach up to 0.8, transfer the culture in 50 ml sterile falcon tubes and chilled on ice for 30 min.
5. Pellet the *Agrobacterium* cells for 10 min at 5000 rpm at 4 ºC. Discard the supernatant.
6. Resuspend the pellet in 5.0 mL ice cold 0.1M $MgCl_2$ and incubated for 30 min on ice
7. Centrifuge the tubes for 10 min at 4,000 rpm, 4 ºC. Remove the supernatant and gently resuspend the pellet in ice cold 0.1M calcium chloride.
8. Centrifuge the falcon tubes for 10 min at 4,000 rpm, 4 ºC. Discard the supernatant.
9. Resuspend the pellet in 500 µl ice cold 0.1M CaCl2 on ice. Add 30% autoclaved glycerol to the resuspended *Agrobacterium* cells and mix well.
10. Aliqout 100 µl *Agrobacterium* GV3101competent cells in 1.5 ml eppendorf tubes. Snap freeze the competent cells snap freeze the tubes in liquid nitrogen or directly proceed to the transformation and store at -80 ºC.

II) Freeze-Thaw Transformation of *A. tumefaciens* GV3101competent cells:

1. Add 500 ng plasmid DNA to 1 tube of competent cells. Hold on ice for 15-30 min if using frozen cells, or 5 min if using freshly prepared cells.
2. Freeze in liquid nitrogen for 5 min.
3. Heat shock in 37 ºC water bath for 5 min, then return to ice for ~5 minutes.
4. Add 1.0 ml of LB to the tube, and then incubate on a 28 ºC rotating shaker for 3-4 hrs.
5. Plate out 50-200 ul of culture on an LB plate containing an appropriate selection agent.
6. Grow up plates for 48hrs at 28 ºC and proceed to colony PCR to confirm transformation.
7. The plasmid DNA from the positive clones can be back transformed into *E. coli* competent cells and further confirmed by plasmid isolation from the positive colonies (*E. coli*) and confirmed by restriction digestion

III) Agrobacterium mediated plant transformation using hypocotyl explant

A general protocol for *Agrobacterium* mediated plant transformation has been given below. Howver, minor modification of the individual steps may be required depending on the individual plant species.

Seed germination

Seeds need to be surface sterilized with 0.1% $HgCl_2$ with vigorous shaking for 5 min followed by five time washing with sterile distilled water. The surface sterilized seeds are placed on half-

strength MS (Murasnige & Skoog, 1962) medium and kept at 25±2^{0}C under a 16/8 hr (day/night) photoperiod for germination.

Pre-culture and co-cultivation of *Brassica juncea*

Hypocotyls of 4-6 day-old seedlings (needs to be optimized) were cut in to 6-7 mm pieces under aseptic condition with the help of sterile scalpel and blade. The explants are pre-cultured for 24-48 h (needs to be optimized) on MS medium supplemented with combination of phytohormones (auxin and cytokinin) viz. BAP, NAA, IAA (regeneration protocol needs to be optimized) and 3.5 mg/ml $AgNO_3$. A single colony of *Agrobacterium* GV3101 bacterial cultures containing binary construct is grown in YEM medium supplemented with appropriate antibiotics for 36 h at 28^{0}C. 200 µl of primary culture is freshly inoculated in 50 ml YEM medium with the same supplements and grown at 28 ^{0}C /180 rpm until the OD_{600} reached 0.4-0.6. The bacterial culture was centrifuged at 6000 rpm for 10 min, pellets are dissolved and diluted 10 times with liquid MS medium. The pre-cultured hypocotyl explants are incubated in *Agrobacterium* suspension for 10 min and then the explants are soaked on sterile paper towels in a laminar air flow for 10-15 min. The explants are transferred back on to the pre-culture medium (co-cultivation medium) and incubated at 25±2 ^{0}C for 24-48 h.

Selection of transformants

Following co-cultivation, explants are washed with sterile distilled water for two times and one time with liquid MS medium containing 250 mg/l cefotaxime followed by air drying on sterile paper towel for elimination of *Agrobacterium* suspension. After washings and drying, the explants are shifted on to the MS medium supplemented regime of phytohormones, 3.5 mg/ml $AgNO_3$ and 250 mg/l cefotaxime for 2-4 days. After 2-4 days explants are again shifted to selective MS medium additionally supplemented with antibiotic for plant selectable marker. The explants were cultured for 20-25 days in selection medium. Emerging green shoots were excised and transferred to culture tubes for shoot elongation. The tubes may contain basal MS medium supplemented only with 250 mg/ml cefotaxime and selective antibiotic. Elongated shoots (after 15-20 days) with normal growth on selective media are transferred to rooting MS medium (half-strength MS + 2 mg/l IBA, 2.5 g/l Phytagel, 250 mg/ml cefotaxime and selective antibiotic) for in vitro rooting. The plantlets obtained were hardened in soilrite, soil and vermicompost mixture (3:1:1), acclimatized and grown to maturity under suitable conditions.

IV) Molecular analysis of the transgenic plants

Presence of transgene, gene expression and other desired effected are needs to be analysed using various molecular techniques and phenotyping.

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Chapter 11 DNA isolation from CRISPR-Cas9 lines

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Principle

Good quality DNA is a prerequisite for all experiments of DNA manipulation. Isolating DNA from plant tissues can be very challenging since the biochemistry between divergent plant species is generally extreme. The structure of the plant cell is more complicated than the animal cell mainly due to cell wall hence DNA cannot be extracted out easily from the plant cell. Polysaccharides and polyphenols are two classes of plant biomolecules in the cell wall that vary widely between species and are very problematic when isolating DNA. Contaminating polysaccharides and polyphenols can interfere with manipulations of DNA following isolation.

All plant DNA extraction protocols comprise of the basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites. This is brought about by disruption of the tissue in a mortar and pestle aided by liquid nitrogen, (adding liquid nitrogen arrests the enzymatic reaction as well as assists in fine crushing of the tissues) and the various components of the homogenization or extraction buffer followed by precipitation and purification methods employed. In the year 1980, Murray & Thompson developed an inexpensive and a simple protocol for the plant DNA extraction with the help of CTAB. The significance of each step and its constituents of DNA extraction protocol are as follows:

A. Extraction-CTAB buffer

This includes a cationic detergent such as cetyl trimethyl ammonium bromide (CTAB) which disrupts the membranes, while additives, such as polyvinylpyrrolidone, can aid in removing polyphenols, a reducing agent such as β-Mercaptoethanol which helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract. A chelating agent such as EDTA which chelates the magnesium ions required for DNase activity, Tris buffer at pH 8 and sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together.

B. Separation of nucleic acid- Chloroform isoamyl alcohol

Nucleic acid solutions commonly contain undesirable contaminants that majorly comprise of proteins. A classic method of purifying DNA from proteins is chloroform isoamyl alcohol extraction by which nucleic acid solution is extracted by successively washing with a volume of chloroform: isoamyl alcohol (24:1). Centrifugation is performed intermittently and the upper aqueous phase is transferred to a new tube while avoiding the interphase. The contaminants are denatured and accumulate in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase.

C. Precipitation -70% Alcohol

Alcohol precipitation is the most commonly used method for nucleic acid precipitation. This requires diluting the nucleic acid with a monovalent salt, adding alcohol to it and mixing gently. The nucleic acid precipitated spontaneously and can be pelleted by centrifugation. The salts and alcohol remnants are removed by washing with 70% alcohol. The most commonly used salts include sodium acetate pH 5.2(final volume 0.3M), sodium chloride (final concentration 0.2M), ammonium acetate (2- 2.5M), lithium chloride (0.8M) and potassium chloride. Ethanol or isopropanol are the standard alcohols used for nucleic acid precipitation.

D. Purification of DNA

The DNA is purified by incubating the nucleic acid solution with RNase A (10mg/ml) at 37°c and reprecipitation following chloroform:isoamylalcohol extraction to remove the RNase A.

E. Resuspending DNA

The nucleic acid pellet can be resuspended in either sterile distilled water or TE buffer (10 mM Tris: 1mM EDTA).

Materials Required

- CTAB buffer:
  2% cetyltrimethylammonium bromide,

  1% polyvinyl pyrrolidone

  100 mM Tris-HCl,

  1.4 M NaCl,

  20 mM EDTA
- Bench top centrifuge (up to 14,000 x g)
- Isopropanol
- 70% Ethanol

- 2 ml and 1.5ml eppendorf tubes
- Mortar and pestle
- Pipettes and sterile tips
- TE Buffer (10 mM Tris, pH 8 and 1 mM EDTA)

Components	**10X stock solution**	**2X working solution**	**Volume for 100mL**
CTAB solution	10%	2%	20ml
NaCl	5M	1.4M	28ml
Tris	1000mM	100mM	10ml
EDTA	500mM	20mM	4ml
PVP	10%	2%	10gm
Mercaptoethanol	2%	0.5-1%	Add prior before use
D/W	–		Up to 100ml final volume

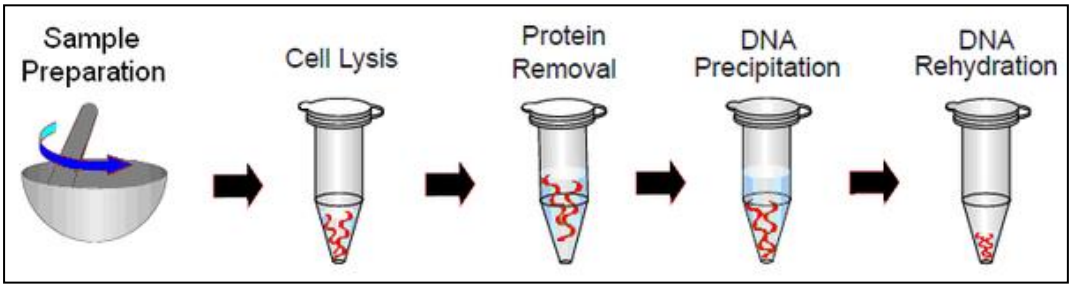
Detailed Protocol

1. Take 200mg of fresh plant tissue and cut it in the small pieces. Add liquid nitrogen to the tissue and roughly grind the sample into the mortar and pestle.
2. Transfer the homogenized tissue in 1mL of CTAB Extraction Buffer containing 10uL of β-ME in each tube (Freshly added). Invert mix several times. Incubate the homogenate to a 60°C bath for 60 minutes.
3. Take out sample and keep them at room temperature till the sample reaches R.T.
4. Add an equal volume of chloroform/isoamyl alcohol (24:1) and mix it well.
5. Centrifuge the sample for 10 min. at 12,000 rpm to separate the phases. Transfer the upper aqueous phase to a new 2mL eppendorf tube.
6. Now add 1 µL of RNaseA solution to the supernatant and incubate at 37°C for 60 minutes.
7. Repeat chloroform/isoamyl alcohol (24:1) step and centrifuge the sample for 10 min. at 12,000 rpm, until the upper phase is clear.
8. Transfer the upper aqueous phase to a new 1.5mL eppendorf tube. Precipitate the DNA by adding 0.6 volume of chilled isopropanol and mix it by gentle inverting. Incubate at room temperature for 5 minutes.

9. Centrifuge the sample at 12,000 rpm for 30 minutes. Decant the supernatant without disturbing the pellet and subsequently wash the pellet with 1mL of chilled 70% ethanol. Centrifuge at 12,000 rpm for 1 minute.

10. Decant the ethanol. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve DNA in 50-80 µl of 0.1x TE buffer (10 mM Tris, pH 8; 1 mM EDTA).

Genomic DNA Isolation Steps



Spectrophotometric Quantification:

Spectrophotometrically quantify total DNA by taking 1 µl of DNA and take OD at 260 nm and 280 nm to determine sample concentration and purity. The A_{260}/A_{280} ratio should be above 1.8 to 2.0. Lower ratio indicates the contaminants with DNA samples such as phenols and proteins. A ratio of A_{260}/A_{230} is used as secondary check for DNA quality in which A230 absorbance detects the presence of contaminants. This ratio should be higher than 260/280 ratio for high quality DNA.

Results

DNA extracted from wildtype/transgenic/mutant lines will be used in subsequent experiments. After extraction, DNA is quantified using NanoDrop. Equal concentration of DNA (2µg) with 6x DNA loading dye is loaded on 1% agarose gel.

Agarose gel electrophoresis of plant genomic DNA. Lane 1-5, gDNA isolated from different mutant lines.

Conclusion

Plant DNA extraction is tedious and very challenging technique. The variability in the structure of the cell wall and the level of secondary metabolites into plant restricts DNA extraction protocol. CTAB extraction buffer is recommended for any type of plant sample; however, modifications are always required.

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Chapter 12 Total RNA isolation by Guanidinium isothiocyanate/TRIzol method

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The isolation of high calibre RNA is imperative in plant molecular biology. The quality of RNA determines the authenticity of downstream processes. While getting high-quality genomic extractions from plants is not trivial, it is even more challenging to consistently obtain sufficient quantity of pure RNA for RNA-Seq (transcriptome) or chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) experiments and Quantitative Real Time PCR (qPCR) analysis. Owing to omnipresence of RNAase and higher degree of instability, RNA is more delicate to manipulate than genomic DNA.RNA is mostly single stranded, often contains ribose sugar that carries 2' hydroxyl group that makes the RNA more prone to hydrolysis than genomic DNA.

It is more complicated to obtain high quality RNA especially when plant samples contain high level of RNAase, large quantities of polysaccharides, low concentration of nucleic acid (high water content), different types of phenolics like tannin and fibrous tissues such as lignin (wood), that are difficult to remove and lysis [1]. The phenolic substances (*i.e.* Polysachharides and polyphenols) bind irreversibly with proteins and nucleic acids, leading to oxidation and degradation that makes it unsuitable for downstream applications. Currently, there are numerous methods of extracting out pure RNA but the most commonly used method is Guanidinium isothiocyanate, also commonly known as TRIzol method. **TRIzol** reagent is a monophasic acid-guanidinium-phenol based reagent ideally designedchemical solution used in the extraction of DNA, RNA, and proteins from cells. The solution was initially used and published by PiotrChomczyński and Sacchi, N. in 1987[2]. TRIzol is the brand name of guanidiniumthiocyanate from the Ambion part of Life Technologies, [3].

Principle

The low pH (acidic) of TRIzol® controls the separation of RNA from DNA and protein, while a high pH can cause RNA and DNA to be isolated together. The guanidinium salt serves as a chaotropic agent to denature proteins as well as inhibit RNAase activity and the phenol (commonly indicated as a pink colour) is an organic compound also used to extract nucleic acids and proteins. After solubilization and homogenization of samples in TRIzol®, the RNA, DNA and protein are differentially extracted by the addition of a phase separation reagent (chloroform). The solution separates the RNA away from DNA and protein into different layers

(Figure 1). An upper, clear aqueous phase mainly contains RNA, and the middle interphase and lower organic phase contains DNA, proteins and lipids. Subsequently, the RNA in the upper aqueous phase is then collected by alcohol-based precipitation. After lengthy incubation and centrifugation steps, the resulting white pellet (if any) is then washed in an ethanol solution, air-dried and resuspended in the final sample buffer.

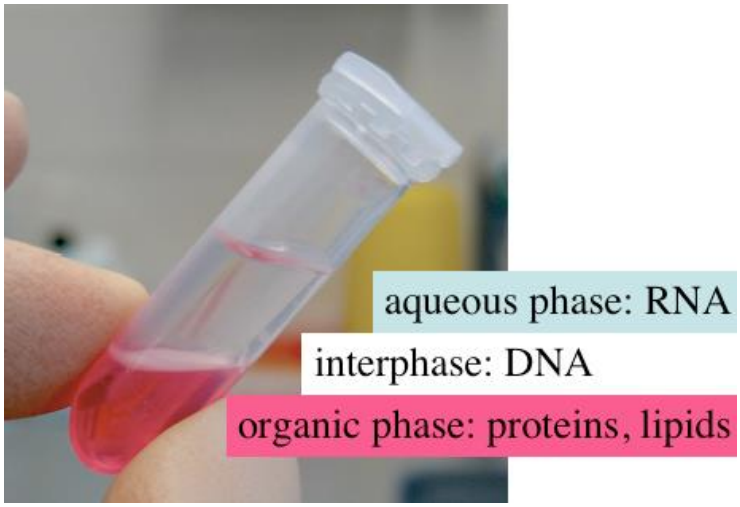

Figure 1: Phase separation during RNA isolation using TRIzol Reagent.

WARNINGS / PRECAUTIONS

A. TRIzol reagent is toxic when in contact with skin and if swallowed. It will cause burns. Be sure to wear a lab coat, gloves and safety glasses when working with TRIzol reagent. If in contact with skin, wash immediately with plenty of soap and water. Work in a chemical fume hood.

B. To prevent RNase contamination, always wear gloves and change them frequently. Also, use sterile, disposable plasticware and pipettes dedicated strictly to RNA work to prevent cross-contamination with RNases from shared equipment.

C. RNA is extremely susceptible to degradation by ribonucleases that are ubiquitous in the environment. To ensure preservation of target RNA or RNA probes, special precautions are needed. Records are maintained to show that RNase-free conditions (i.e. wiping the lab areas with RNaseZAP) are met, with corrective action if conditions are not met.

D. Always use gloves and eye protection. Avoid contact with skin or clothing. Use in a chemical hood. Avoid breathing vapor.

A. Required reagents:

DEPC-treated water (Ambion)

TRIzol Reagent (Invitrogen)

70% ethanol (Molecular Grade)

Isopropyl alcohol

B. Equipment's and supplies:

Refrigerated centrifuge

Autoclaved mortar and Pestle

Micropipettors

Autoclaved tips

Vortex mixer

Powder-free gloves

Autoclaved centrifuge tubes

Autoclaved spatula

Liquid Nitrogen

C. Grinding:

Grind tissue samples in prechilled mortar and pestle with the help of liquid nitrogen or in 1 ml of TRIzol reagent per 100 to 200 mg of tissue. Sample volume should not exceed 10% of the volume of TRIzol Reagent used for grinding. Carefully transfer fine crushed powder with the help of spatula in an autoclaved centrifuge tube. Vortex thoroughly to facilitate complete mixing and cell wall lysis.

D. Phase Separation:

Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely and invert mix sample 5-6 times for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into fresh tube. Measure the volume of the aqueous phase (The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization).

E. RNA Precipitation:

Precipitate RNA from the upper aqueous phase by mixing 0.6 ml of isopropyl alcohol per 1 ml of TRIzol reagent used. Incubate samples at 15 to 30° C for 10 minutes and centrifuge at not more than 12,000 X g for 10 minutes at 2 to 4°C. The RNA precipitate, often invisible before centrifugation, forms a translucent gel like pellet on the side and bottom of the tube.

F. RNA Wash:

Decant the supernatant completely. Wash the RNA pellet once with 70% ethanol, adding at least 1 ml of 70% ethanol per 1 ml of TRIZOL Reagent. Resuspend the RNA pellet by gentle tapping and centrifuge at maximum 7,500 X g for 5 minutes at 2 to 4°C. Repeat above washing procedure once. Remove all leftover ethanol.

G. Re-dissolving RNA:

Air-dry or vacuum dry RNA pellet for15 to 30 minutes. When the pellet is dry, there must be no visible ethanol in the tube. It is important not to over dry the RNA pellet as this will greatly decrease its solubility. Partially dissolved RNA samples have an A_{260}/A_{280} ratio < 1.6. Resuspend the RNA pellet in 30-50 µL of nuclease-free water by pipetting up and down gently and incubating in a 60°C water bath for 10 minutes (can incubate longer if necessary, up to one hour).

H. DNase treatment of RNA:

1. Add 0.1 volume (5µL) of 10X DNase I buffer and 1 µL of DNase I (2 units) to the RNA. Mix gently by flicking tube (DO NOT VORTEX) and incubate in a 37°C water bath for 20 minutes.

2. Add 0.1 volume of DNAse Inactivation Reagent (6µL) to the RNA. (Make sure to vortex the DNAse Inactivation Reagent before addition to the RNA.) Flick (do not vortex) the RNA tube to disperse the reagent. Incubate for 90 seconds at room temperature. Flick the tube once more during the incubation to redisperse the DNase Inactivation Reagent.

3. Centrifuge at 10,000 x g for 1 minute at room temperature. Transfer the supernatant RNA solution to a fresh 1.5 mL micro centrifuge tube.

I. Spectrophotometric Quantification:

Spectrophotometrically quantify total RNA by taking 1 µl of RNA and take OD at 260 nm and 280 nm to determine sample concentration and purity. The A_{260}/A_{280} ratio should be above 1.9. Apply the convention that 1 OD at 260 equals 40 µg /ml RNA.

J. Qualitative Analysis:

For assessing the quality of RNA, 500ng-1µg of total RNA (as per nanodrop reading) is loaded onto 1% Agarose TBE gel (Figure 2).

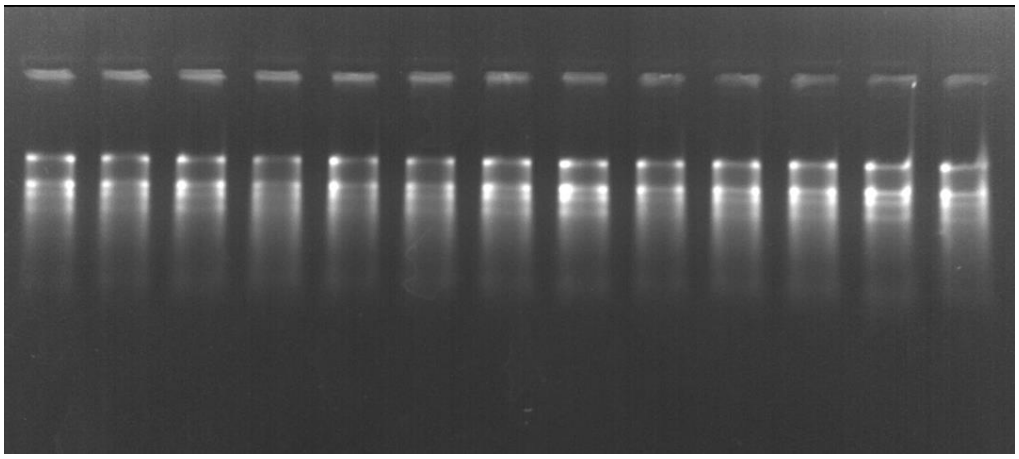

Figure 2: Total RNA quantification on 1% Agarose TBE gel.

K. Storage:

For long term storage, RNA is stored in a -80°C freezer.

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TRIzol reagent literature, Invitrogen Life Technologies.

Chapter 13

Identification of Genome Edited Mutants using PCR-Restriction Fragment Length Polymorphism and RGEN-RFLP

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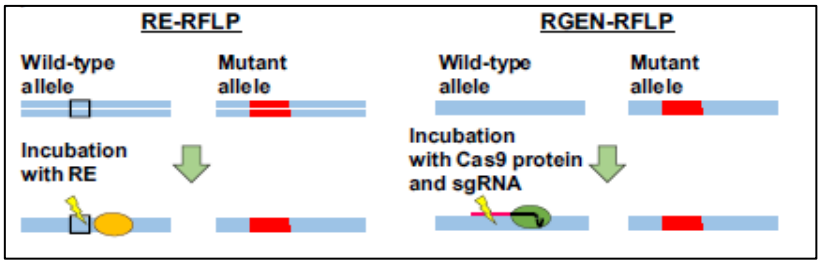
Principle

CRISPR/Cas9 Genome editing has emerged as a promising tool for genome editing in plants using guideRNAs. It is a robust, effective, affordable and easy to engineer editing technique where multiple genes can be targeted. However, genotyping is required for detecting these genetic mutants. Various methods are available for genotyping viz. fluorescent-PCR, deep sequencing, T7 endonuclease-based assay, restriction endonuclease (RE)-based assay, PCR-RFLP and RGEN (RNA guided engineered nucleases) - RFLP assay. PCR-RFLP is a widely used method which is less expensive and can accurately detect the somatic mutations. It allows to distinguish between mutant and wild type sequences via destructing or generating enzyme restriction sites through PCR and subsequent electrophoresis separation of differential fragments. Compared to other methods of genotyping, PCR-RFLP do not require expensive equipment and are highly reproducible and sensitive method. The principle of PCR-RFLP is very simple: A restriction enzyme or Cas9 RNP cleaves homoduplex PCR products from wild-type alleles, but not homoduplex PCR products from mutant alleles.

PCR-RFLP is one of the oldest, convenient, inexpensive and widely used methods of genotyping but is limited by the availability of appropriate restriction endonuclease sites. To avoid this limitation, a novel method of detecting and quantifying engineered nucleases-induce mutations has been developed using RGEN in RFLP analysis. RGEN-RFLP analysis can distinguish homozygous mutant clones with identical biallelic indel sequences from wild type clones and is not limited by sequence polymorphism near the nuclease target sites. Moreover, RGEN-RFLP can also be used to genotype naturally occurring indels. RGENs recognize and cleave DNA in a targeted manner using two separate components, a small RNA component CRISPR RNA (crRNA)or single chain guide RNA (sgRNA) and the Cas9 protein. crRNA or sgRNA hybridizes with a 20 base pair long target sequence and Cas9 recognizes the PAM sequences, NGG (or NAG) trinucleotide sequence. Thus, an RGEN site can be shown as 5'-X_{20}NGG-3', where X_{20} corresponds to crRNA sequence and N is any base.

In principle, genomic PCR products from wild-type alleles are completely cleaved by a restriction enzyme or Cas9 RNP, whereas those from mutant alleles can no longer be cleaved by these enzymes owing to the alteration in the target site by NHEJ or MMEJ repair.



Schematic representation of PCR-RFLP and RGEN-RFLP assay

Materials Required

- Nuclease free microcentrifuge tubes- 0.5 ml, 1.5 ml, 2 mL; Pipettes -10 µL, 200 µL, 1 ml; Pipette tips- 1 mL, 200 µL and 10 µL; Nuclease free PCR tubes
- Instruments: PCR machine, Dry bath, Water bath, Gel electrophoresis unit, Microcentrifuge, Gel documentation unit, Nanodrop, sonicator.
- Reagents and components: Genomic DNA, Agrose, Restriction enzyme, Restriction buffer specific to RE, 10X Taq Buffer, dNTP Mix 2 mM each, Forward and Reverse primer, 25 mM $MgCl_2$, Taq DNA Polymerase, Phusion polymerase (New England Biolabs), Nuclease free water, IPTG, Tris (pH 8.0), NaCl, imidazole, PMSF, HEPES (pH 7.5), KCl, DTT and 10% glycerol
- pET-28a expression vector, BL21(DE3) culture cells

Detailed Protocol

PCR-RFLP assay

1. Amplify the target region using PCR with < 30 cycles to prevent heteroduplex formation with gene specific primers.

PCR reaction:

S. No.	Components	Volume added	Final Concentration
1	Nuclease free water	To 20 µl	
2	10X Taq Buffer	2.0 µl	1 X
3	25 mM dNTP Mix	1.2 µl	
4	25 mM $MgCl_2$	1.6 µl	2.5 mM
5	Forward primer	2.0 µl	0.1-1 µM
6	Reverse primer	2.0 µl	0.1-1 µM

Reaction mix is given a short spin at this point			
7	Taq DNA Polymerase (*3unit/ µl)		1 U
8	Template DNA	50-100 ng	

Gently vortex the samples and spin down.

S. No.	Steps	Temperature (°C)	Time	Number of cycles
1	Initial denaturation	94	5 min	1
2	Denaturation	94	30 s	30
3	Annealing	Tm	30 s	
4	Extension	72	1 min/kb	
5	Final Extension	72	5 min	1
6	Hold	4		

2. Purify PCR product or amplicon using PCR purification kit.
3. Quantify the PCR products (5 µl) on 1.75% agarose gels supplemented with 0.5 µg of ethidium bromide per ml at constant current in Tris-borate-EDTA buffer with appropriate molecular size marker in first lane.
4. Expected nucleotide sequences of the PCR product can be restriction mapped with the Mapdraw program of DNASTAR (NY, USA).

Setting up of restriction enzyme digestion reaction:

S.No.	Components	Volume	Example
1	Nuclease free water	To 20 µl	11 µl
2	Buffer (10 X)	2 µl (1 X)	2 µl
3	DNA	500 ng –1 µg	5 µl
Reaction mix is given a short spin at this point			
5	Restriction Enzyme	20 unit	2 µl
	Total	20 µl	20 µl

The tubes were incubated at 37°C for approximately 4 h.

5. The digested PCR products are then separated on 2.5% agarose gel to distinguish between mutant allele from wild type allele.

RGEN-RFLP assay

6. RGEN components: New RGEN plasmids are easily prepared by cloning 20-bp guide DNA sequences in a vector that encodes either crRNA or sgRNA.

RGENs can be made without cloning: crRNA or sgRNA can be prepared by annealing two complementary oligonucleotides followed by *in vitro* transcription using MEGA shortcript T7 kit (Ambion) according to the manufacturer's instruction.

6.1 Transcribed RNAs are resolved on an 8% denaturing urea-PAGE gel.

6.2 Gel slice containing RNA is cut out and transferred to elution buffer. RNA is recovered in nuclease-free water using gel elution kit followed by phenol:chloroform extraction, chloroform extraction and ethanol precipitation. Purified RNA is quantified by Nanodrop reading.

6.3 Templates for crRNA can be prepared by annealing an oligonucleotide X20 (target sequence) and its complementary oligonucleotide. The template for tracrRNA is synthesized by extension of forward and reverse oligonucleotides using Phusion polymerase (New England Biolabs).

7. Recombinant Cas9 protein: Recombinant Cas9 protein can be purchased from ToolGen, Inc. or purified from *E. coli.*

7.1 The Cas9 DNA construct that encodes *S. pyogenes* Cas9 fused to the His6-tag at the C terminus is inserted in the pET-28a expression vector. The recombinant Cas9 protein is expressed in *E. coli* strain BL21(DE3) cultured in LB medium at 25 °C for 4 h after induction with 1mM IPTG.

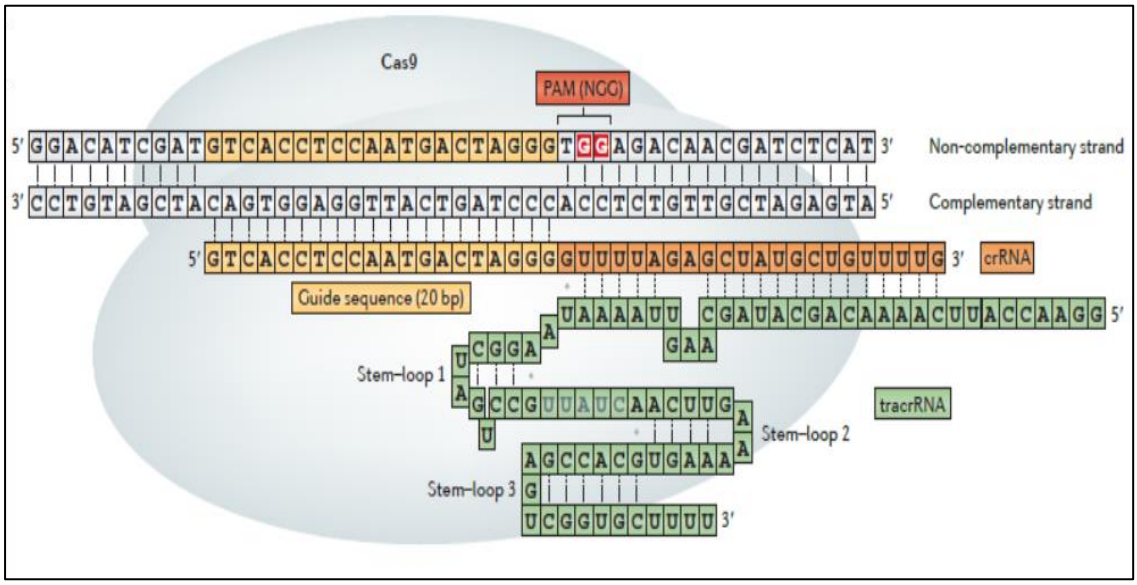
7.2 Cells are harvested and resuspended in buffer containing 20mM Tris (pH 8.0), 500mM NaCl, 5mM imidazole and 1mM PMSF. Cells are then frozen in liquid nitrogen, thawed at 4 °C and sonicated. After centrifugation, Cas9 protein in the lysate is bound to Ni-NTA agarose resin (Qiagen), washed with buffer containing 20mM Tris (pH 8.0), 500mM NaCl and 20mM imidazole, and eluted with buffer containing 20mM Tris (pH 8.0), 500mM NaCl and 250mM imidazole.

7.3 Purified Cas9 protein is dialyzed against 20mM HEPES (pH 7.5), 150mM KCl, 1mM DTT and 10% glycerol and analysed by SDS–PAGE.

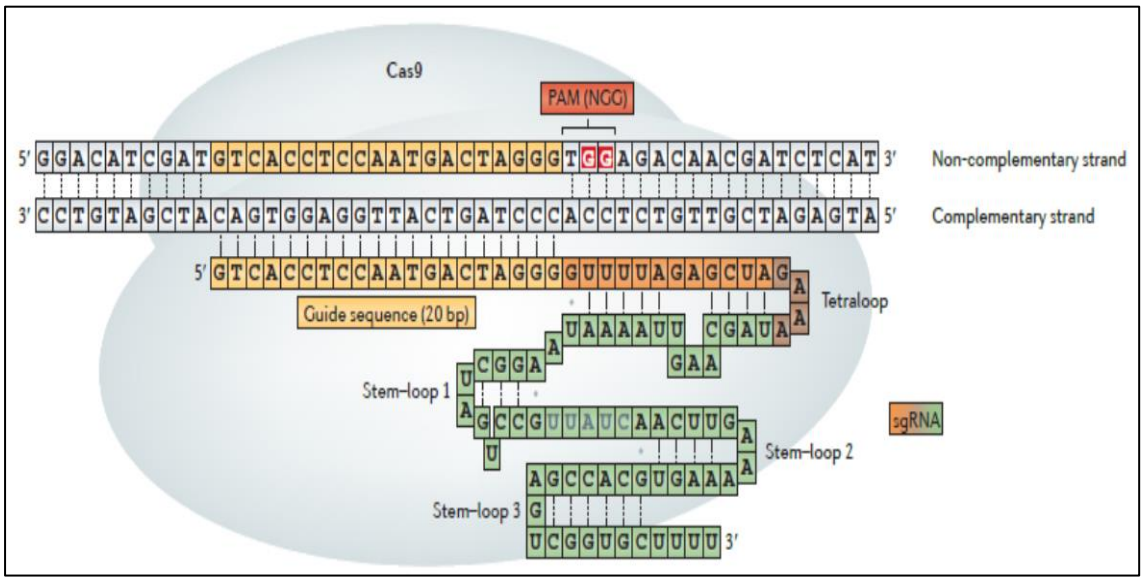
8. RGEN-RFLP assay: PCR products (100–150 ng) can be incubated for 60 min at 37 °C with optimized concentrations of Cas9 protein, tracrRNA, crRNA in 10 ml NEB buffer 3 (1X).

9. After the cleavage reaction, RNaseA (4mg) is added, and the reaction mixture is incubated for 30 min at 37 °C to remove RNA. Reactions are stopped with 6X stop solution buffer containing 30% glycerol, 1.2% SDS and 100mM EDTA.

10. Products are resolved with 1–2.5% agarose gel electrophoresis and can be visualized with EtBr staining.



RGEN comprising of crRNA, tracrRNA and Cas9 which form the dualRNA-Cas9. The guide sequence in crRNA is complementary to a 20-bp target DNA sequence (Protospacer) which is next to the 5′-NGG-3′ sequence (PAM).



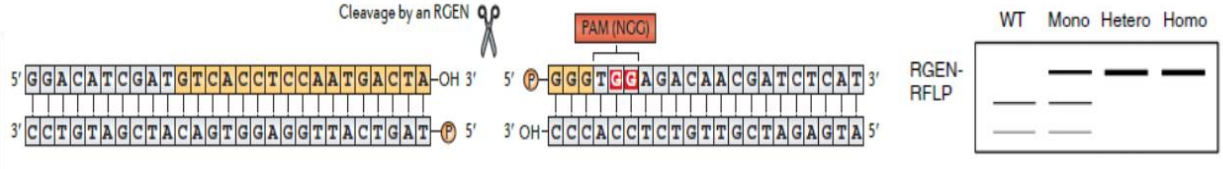
RGEN comprising of Cas9 and single chain guide (sgRNA). The guide sequence in sgRNA is complementary to a 20-bp target DNA sequence (Protospacer) which is next to the 5′-NGG-3′ sequence (PAM).

Table 1: Resources for RNA guided engineered nucleases

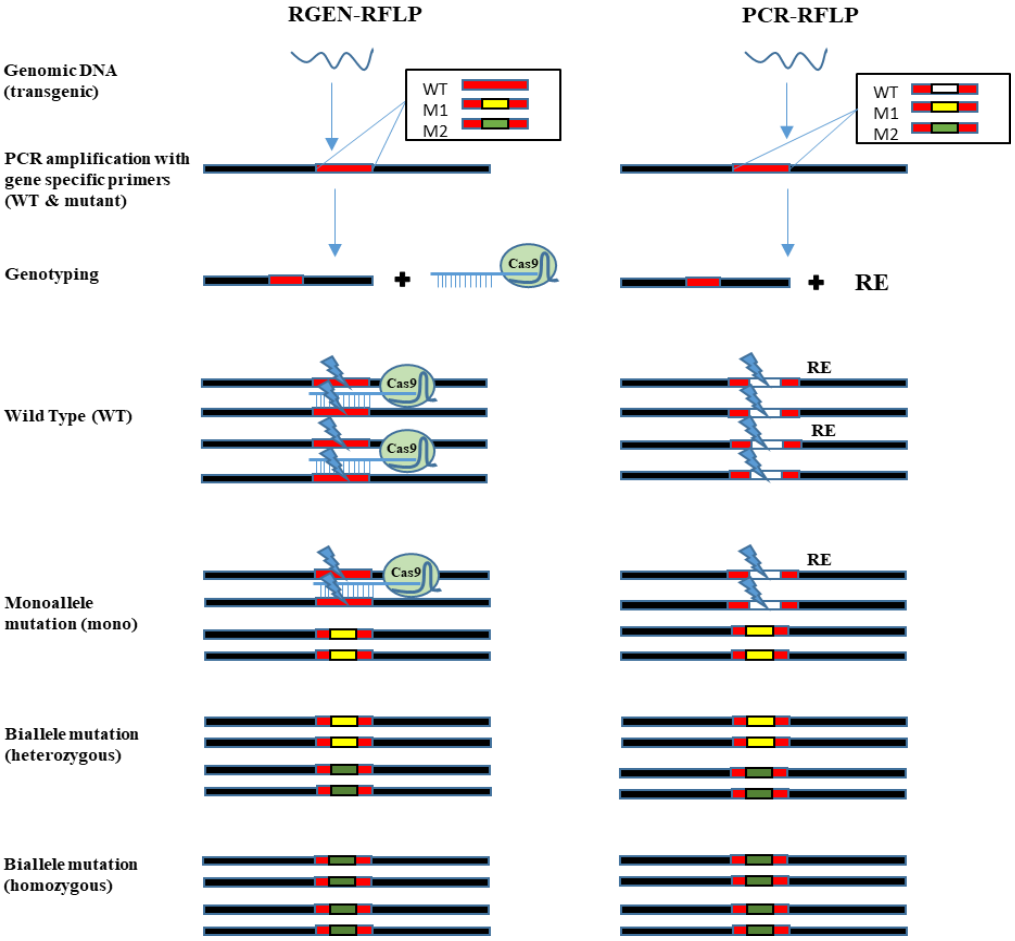
	Online Resources for nuclease design	**Suppliers**	
		Non-Profit Organizations	**Companies**
RGEN	E-CRISP	Addgene	Life Technologies
	Genome engineering resources		Sigma-Aldrich
	RGEN tools		System Biosciences
	ZiFiT Targeter software		ToolGen
			Transposagen Biopharmaceuticals

Each black line represents RGEN-target sites for RFLP. The PAM sequence recognized by Cas9 is underlined.

Results



Target DNA cleaved by an RGEN and expected result of RGEN digestion resolved by electrophoresis.



Conceptual diagrammatic illustration of PCR-RFLP and RGEN-RFLP assay. In RGEN-RFLP analysis, in-vitro transcribed gRNA and purified Cas9 protein is used to detect mutation while in PCR-RFLP specific RE are used to identify indels. This method can differentiate WT and mutants but cannot differentiate biallelic heterozygous and homozygous mutations. Yellow and green boxes represent insertion/ deletion mutations generated by NHEJ.

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Chapter 14 Identification of genome edited mutants by Heteroduplex assay-T7 nuclease assay

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Background

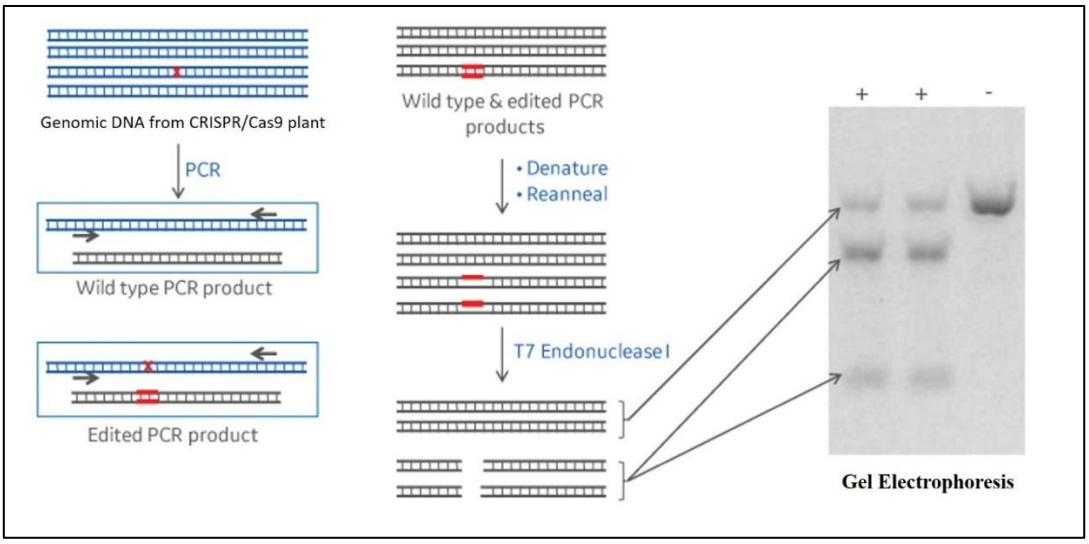
The CRISPR/Cas9, one of the key techniques used for targeted mutagenesis. This genome editing method involves random repair of an induced double-stranded DNA break through the non-homologous end joining (NHEJ) repair pathway, resulting in small insertions/deletions. Since genome editing does not target all loci with similar efficiencies therefore, it becomes very important to evaluate the mutation hit-rate at a given locus. Multiple methods have been developed to enable the detection of such mutations. One of the most direct method utilized to identify the sequence changes/differences is through reading the DNA sequence with traditional and high throughput DNA sequencing methods. However, this method is costly and time consuming. Other widely used non-sequencing methods depend on physical properties of DNA, for example melting temperature-based systems such as Single-stranded conformational polymorphism analysis (SSCP) and Denaturing high-performance liquid chromatography (DHPLC). These techniques are limited to indicate the presence of polymorphism(s), but do not easily yield the location of a mutation within a DNA sequence. Therefore, this must be followed with additional techniques in order to pinpoint the mutation or map multiple mutations in the same fragment.

Enzymatic mismatch cleavage assays exploit the properties of mismatch-specific endonucleases to detect and cleave mismatches. These methods are simple to run using standard laboratory techniques and equipment, and can detect polymorphisms, single base pair mismatches, and insertions and deletions at low frequencies. Several such enzymes have been discovered (including CEL I, T4 endonuclease VII, Endonuclease V, T7 endonuclease I).

One of the commonly used enzymatic assay to determine such mutation is T7 endonuclease I (T7EI)–based heteroduplex cleavage assay. This enzymatic assay provides a rapid screening step prior to DNA sequencing. By isolating candidates likely to contain the desired zygosity for the edited gene, this screening method can decrease the number of clones required for DNA sequencing.

Principle

The principle behind T7 Endonuclease I assay is its ability to recognize and cleave non-perfectly matched DNA. For this assay, first of all, the genomic DNA is extracted from the plants whose genome is targeted using CRISPR/Cas9. Then the amplicons encompassing targeted sites are amplified from genomic DNA. If a non-homologous end joining (NHEJ) repair event following CRISPR-Cas9 cleavage has introduced a mutation, denaturing and annealing will form a fraction of heteroduplexes of mutant and wildtype PCR amplicons. T7 endonuclease I will recognize and cleave DNA mismatches in those heteroduplexes. Running the cleavage products on a polyacrylamide or agarose gel, will resolve full length and cleavage products. The intensity of the respective bands will allow to calculate the gene editing percentage that has occurred (Figure 1).



Schematic and example of a DNA mismatch detection assay. Genomic DNA (blue) from edited cells contains wild type and edited DNA (mutation in red). PCR amplification around the editing site generates wild type and edited PCR products (black). Denaturing and reannealing of these products generates mismatches that are cleaved by the T7EI nuclease. Running these PCR products on a gel resolves full length DNA and cleavage products. Gel shows untreated (-) and cells edited with Cas9 (+).

Materials required

1. Nuclease free microfuge tubes-0.5 ml, 1.5 ml, 2ml; Pipettes-10µl, 200 µl, 1ml; Pipette tips- 1ml, 200 µl and 10 µl; Nuclease free PCR tubes and Nuclease free water; Gloves to wear during all steps
2. Instruments: Thermocycler with programmable temperature ramp rate; Apparatus to quantitate DNA (spectrophotometer of fluorometer); Apparatus to analyze DNA fragments (e.g. Agilent Bioanalyzer or standard agarose gel electrophoresis)

3. Reagents and Components: Q5® Hot Start High-Fidelity 2X Master Mix; T7 Endonuclease I; 0.5 M EDTA pH 8.0; Purified genomic DNA from targeted and control plants; PCR primers to amplify ~700bp region containing the target site; dNTPs; Optional: DNA purification system (e.g. High Pure PCR Product Purification Kit)

Detailed Protocol

1. **PCR Amplification**
   - Set up a 50 µl PCR reaction using ~100 ng of genomic DNA as a template. For each amplicon set up 3 PCR reactions using the following templates
   - gDNA from targeted plants
   - gDNA from negative control
   - water (i.e. no template control)

PCR using Q5 High-Fidelity DNA Polymerase

COMPONENT	50 µl REACTION	FINAL CONCENTRATION
Q5 Hot Start High-Fidelity 2X Master Mix	25 µl	1X
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA	Variable	100 ng total
Nuclease-free water	To 50 µl	

- Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Transfer PCR tubes to a PCR machine and begin thermocycling.

Cycling Conditions

Step	Temperature	Time
Initial denaturation	98°C	30 seconds
35 Cycles	98°C	5 seconds
	50-72°C	10 seconds
	72°C	20 seconds
Final extension	72°C	2 minutes
Hold	4-10°C	

**Note: Q5 Hot Start High-Fidelity 2X Master Mix does not require a separate activation step. Standard Q5 cycling conditions are recommended.*

- Analyze a small amount of the of the PCR product to verify size and appropriate amplification on 1% agarose gel.
- Purify the PCR reaction using 90 µl of Ampure XP beads following the manufacturer's recommendations. Other PCR purification systems (e.g. Monarch PCR & DNA Clean Up Kit, or Zymo DNA Clean and Concentrator™) are acceptable.
- Elute PCR products in 30 µl of water, recovering 25 µl.
- Measure the concentration of the purified PCR products.

2. Heteroduplex formation

a) Assemble reactions as follows

Component	**19µl Annealing reaction**
DNA (Purified PCR product)	200ng
10X NEBuffer	2 µl
Nuclease-free water	To 19µl

b) Anneal the PCR products in a thermocycler using the following conditions

Hybridization Conditions

Step	**Temperature**	**Ramp rate**	**Time**
Initial denaturation	95°C		5 minutes
Annealing	95-85°C	-2°C/second	
	85-25°C	-0.1°C/second	
Hold	4°C		Hold

- Use the annealed PCR product for T7 Endonuclease I digestion.

3. **T7 Endonuclease I digestion**

Components	**20µl Reaction**
Annealed PCR product	19µl
T7 Endonuclease I	1µl
Incubation Time	15 minutes
Incubation temperature	37°C

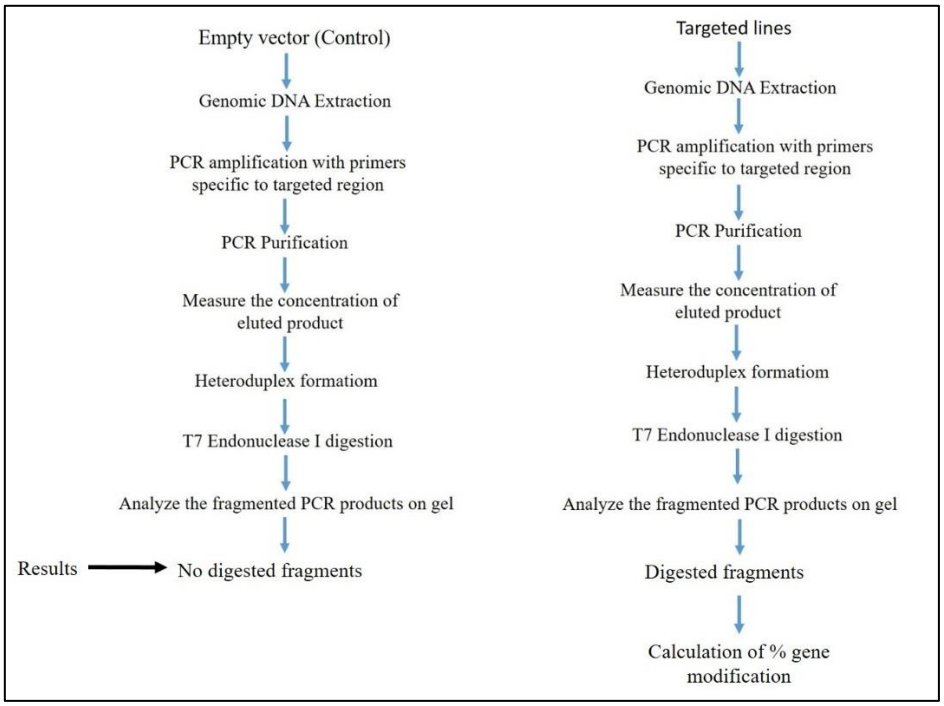
- Stop the reaction by adding 1.5 µl of 0.25 M EDTA.
- Purify the reaction using 36 µl of Ampure XP beads according to the manufacturer's suggestion. This step is optional since 1 µl of the reaction will not interfere with analysis on an Agilent Bioanalyzer using DNA1000 reagents.
- Elute the DNA fragments in 20 µl of water, recovering 15 µl.

❖ **Analysis:**

- Analyze the fragmented PCR products and determine the percent of nuclease-specific cleavage products (fraction cleaved)
- Calculate the estimated gene modification using the following formula:

  % gene modification = 100 x (1 – (1- fraction cleaved)$^{1/2}$)

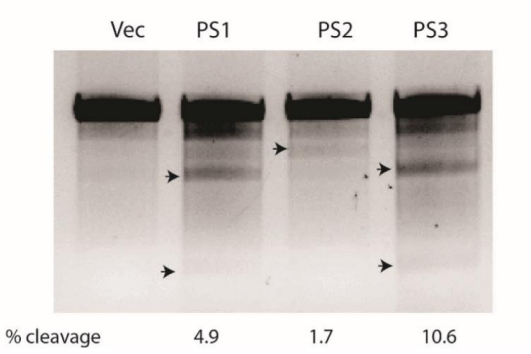
Flow Chart



Results and Conclusion

1. In the above example, three target lines *viz.*, PS1/2/3 were digested with T7 Endonuclease I and digested fragments were detected in all the targeted samples but not in the empty vector control.

2. Based on the ratio of T7E1-digested and -undigested DNAs, the percentages of targeted mutations in OsMPK5 were calculated using % gene modification formula and found to be 4.9%, 1.7%, and 10.6% for PS1, PS2, and PS3 samples. The intensity of DNA bands was calculated using Image J (http://rsbweb. nih.gov/ij/) (Xie and Yang, 2013).



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Chapter 15

Sequence Analysis of decoding Sanger sequencing chromatograms from CRISPR-Cas9 Induced Mutations

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To validate the outcome of CRISPR-mediated genome editing, it is necessary to confirm the targeted mutations and determine the mutated sequences for further characterization. A variety of methods, including T7E1, qPCR assay, restriction enzyme site-based assay, high-resolution melting curve assay, and Surveyor nuclease assay, have been applied to screen mutations in target sites and measure the editing efficiency. However, these methods are not applicable for all targeted mutation types neither can resolve the mutation sequences. Although deep sequencing-based methods is high-throughput and capable of detecting rare mutations in chimeric mutants, it is costly and time-consuming, thus not convenient enough for routine use. CRISPR/Cas9- based genome editing generates mostly uniform mutations (i.e., non-chimeric mutations, including biallelic, homozygous, and heterozygous mutations).

Principle

In CRISPR-Cas9 mediated genome editing, mutations usually occur at positions adjacent to the cleavage site. If the decoded mutations are far away from the target(s), the results would be unreliable. To decode the chromatograms several methods like DSDeCodeM ((http://skl.scau.edu.cn/)) and CRISPR-ID (http://crispid.gbiomed.kuleuven.be/) automatic web-based tools were developed and used to decode the degenerate or super imposed chromatograms and easily to find out the insertions or deletions and identifying zygosity of genome edited plants or mutated cell lines i.e. genotyping work of CRISPR-induced mutants. In both the cases sequencing trace files (ABI and SCF files) were used for analysis. DSDecodeM can be used to look for any kind of mutation on up to 2 different alleles. CRISP-ID directly reads and is the first application with the ability to de-convolute the overlapping spectra from three different alleles, providing a robust and easy to use clone identification tool using direct standard Sanger sequencing of PCR products from cell line clones or patient material, without bacterial sub-cloning. CRISP-ID can be used to look for any kind of indel on up to 3 different alleles. CRISP-ID was designed to determine the size and locus of indels and does this by trying to determine the exact sequences as accurately as possible. Poor chromatograms or weaker signals, the accuracy of base calling can be compromised. Poor sequences can be trimmed or adjusting the % background cut-off value.

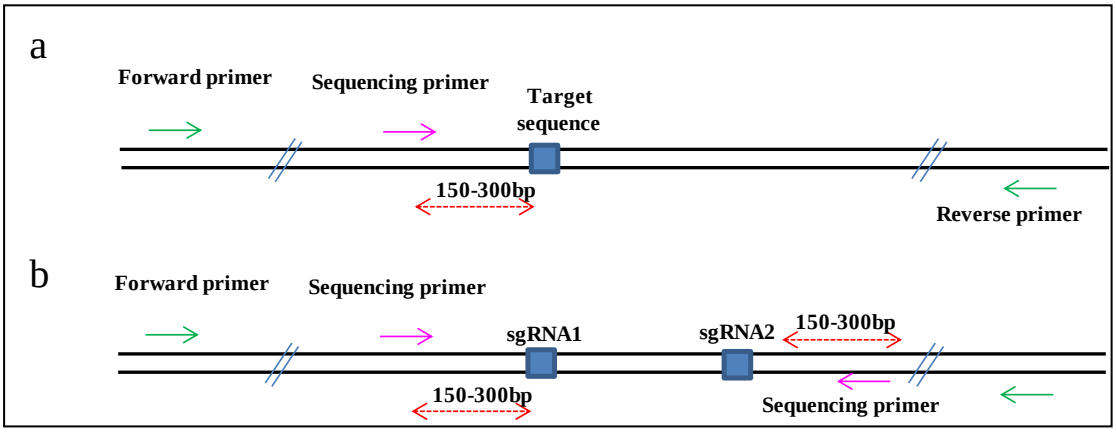
Materials required

1. Genomic DNA samples from plants transformed with CRISPR constructs.
2. PCR reagents including Taq DNA polymerase, dNTPs, primers, and PCR buffer.
3. DNA gel purification kit.

Detailed protocol

Manual Designing of Primers for PCR and Sequencing

1. Design specific PCR primers: Generally, a primer pair should be about 200–400 bp flanking the targeted sites in the genome. For two or more closely positioned target sites, design PCR primers to produce longer (e.g., about 2–3 kb) amplicons containing these target sites (Figure 1).
2. Design nest sequencing primers to obtain high-quality sequencing chromatograms; nest primers, rather than reusing the PCR primers are designed as the sequencing primers, which are located about 150–300 bp away from the targeted sites. If the amplicons contain multiple target sites, design an independent sequencing primer for each site

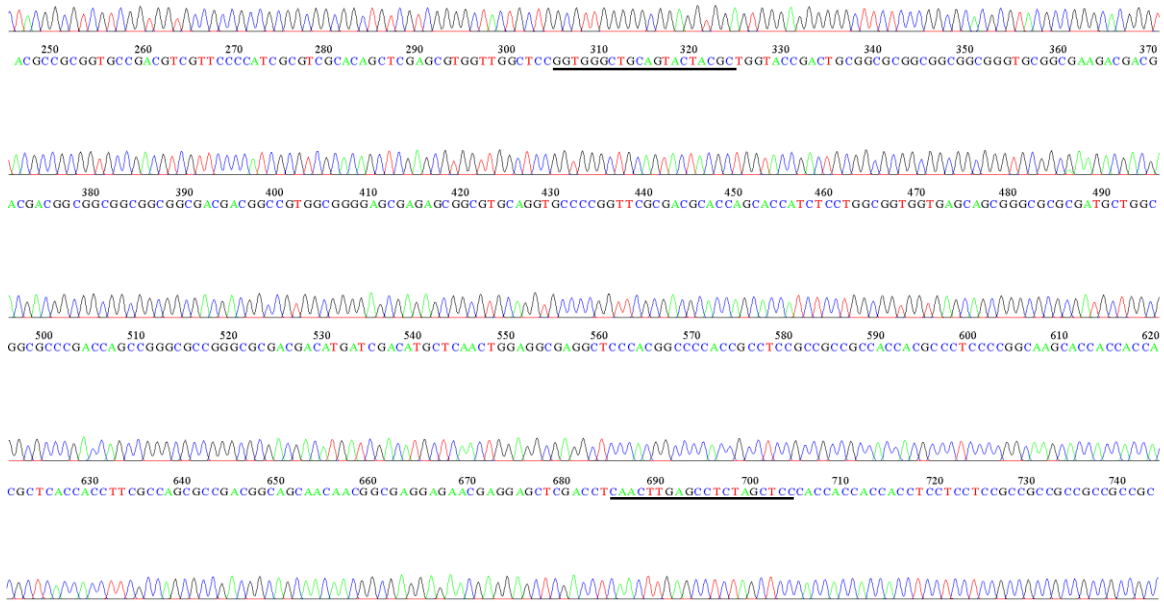


PCR and Sequencing

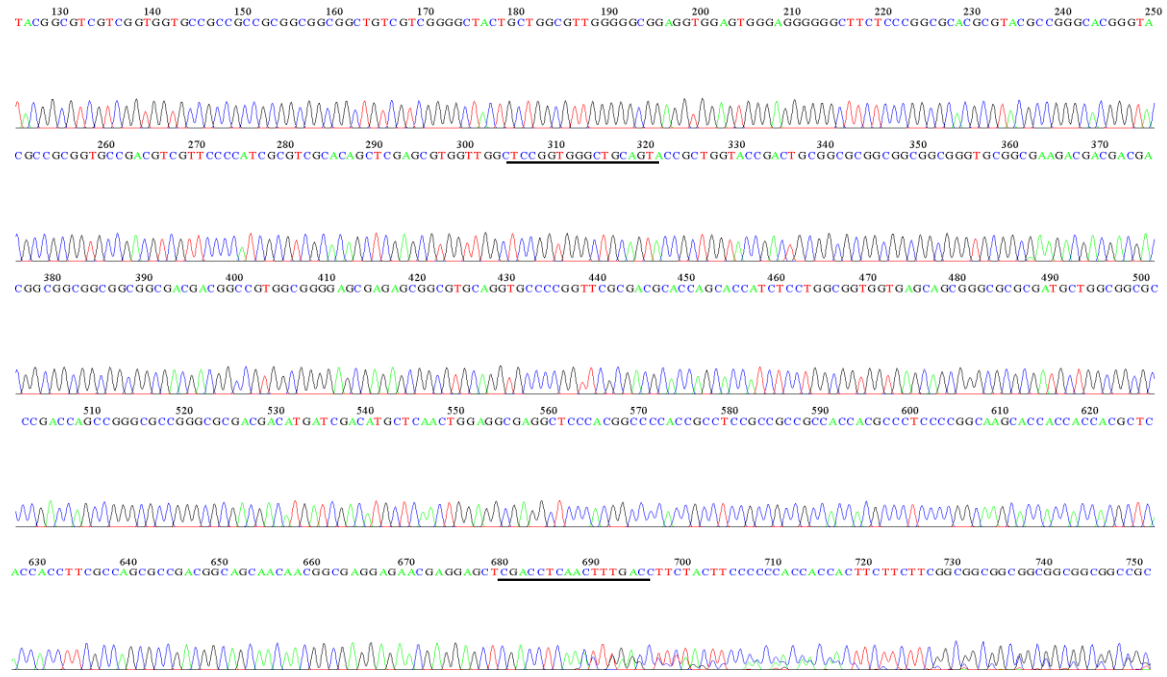
1. Amplify the target site(s)-containing genomic fragment (s) from candidate genome-edited individuals with the designed specific primers following a regular PCR procedure.
2. Purify the PCR products by agarose gel electrophoresis and the use of a gel purification kit.
3. Subject the purified PCR products for Sanger sequencing using internal sequencing primer(s).
4. Reference and mutant sequences were aligned by using multiple sequence alignment tools
5. Superimposed chromatograms can be analysed by using CRISPR-GE web-based decoding tools

An example of decoding targeted mutations using the DSDecodeM Tool

WT reference sequence _ Chromatogram



Mutant sequence _ Chromatogram



Multiple sequence alignment

```
CLUSTAL O(1.2.4) multiple sequence alignment

Mutant      GGAGCTACGGCGTCGTCGGTGGTGCCGCCGCCGCGGCGGCGGCTGTCGTCGGGG-CTACT
Reference   GGAGTTACGGCGTCGTCGGTGGTGCCGCCGCCGCGGCGGCGGCTGTCGTCGGGGCTTACT
            ****  **************************************************   ****

Mutant      GCTGGCGTTGGGGGCGGAGGTGGAGTGGGAGGGGGGCTTCTCCCGGCGCACGCGTACGCC
Reference   GCTGGCGTTGGGGGCGGAGGTGGAGTGGGAGGGGGGCTTCTCCCGGCGCACGCGTACGCC
            ************************************************************

Mutant      GGGCACGGGTACGCCGCGGTGCCGACGTCGTTCCCCATCGCGTCGCACAGCTCGAGCGTG
Reference   GGGCACGGGTACGCCGCGGTGCCGACGTCGTTCCCCATCGCGTCGCACAGCTCGAGCGTG
            ************************************************************

Mutant      GTTGGCTCCGGTGGGCTGCAGTACC--GCTGGTACCGACTGCGGCGCGGCGGCGGCGGGT
Reference   GTTGGCTCCGGTGGGCTGCAGTACTACGCTGGTACCGACTGCGGCGCGGCGGCGGCGGGT
            ************************    ********************************

Mutant      GCGGCGAAGACGACGACGACGGCGGCGGCGGCGGCGACGACGGCCGTGGCGGGGAGCGAG
Reference   GCGGCGAAGACGACGACGACGGCGGCGGCGGCGGCGACGACGGCCGTGGCGGGGAGCGAG
            ************************************************************

Mutant      AGCGGCGTGCAGGTGCCCCGGTTCGCGACGCACCAGCACCATCTCCTGGCGGTGGTGAGC
Reference   AGCGGCGTGCAGGTGCCCCGGTTCGCGACGCACCAGCACCATCTCCTGGCGGTGGTGAGC
            ************************************************************

Mutant      AGCGGGCGCGCGATGCTGGCGGCGCCCGACCAGCCGGGCGCCGGGCGCGACGACATGATC
Reference   AGCGGGCGCGCGATGCTGGCGGCGCCCGACCAGCCGGGCGCCGGGCGCGACGACATGATC
            ************************************************************

Mutant      GACATGCTCAACTGGAGGCGAGGCTCCCACGGCCCCACCGCCTCCGCCGCCGCCACCACG
Reference   GACATGCTCAACTGGAGGCGAGGCTCCCACGGCCCCACCGCCTCCGCCGCCGCCACCACG
            ************************************************************

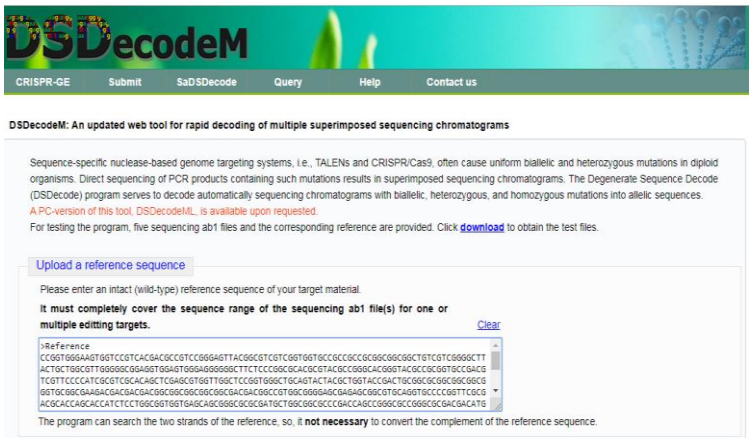
Mutant      CCCTCCCCGGCAAGCACCACCACCACGCTCACCACCTTCGCCAGCGCCGACGGCAGCAAC
Reference   CCCTCCCCGGCAAGCACCACCACCACGCTCACCACCTTCGCCAGCGCCGACGGCAGCAAC
            ************************************************************

Mutant      AACGGCGAGGAGAACGAGGAGCTCGACCTCAACTTTGACCTTCTACTTCCCCCCACCACC
Reference   AACGGCGAGGAGAACGAGGAGCTCGACCTCAACTTGAGCCTCTAGCT-CCCACCACCACC
            ************************************    ***     ** ***  ********

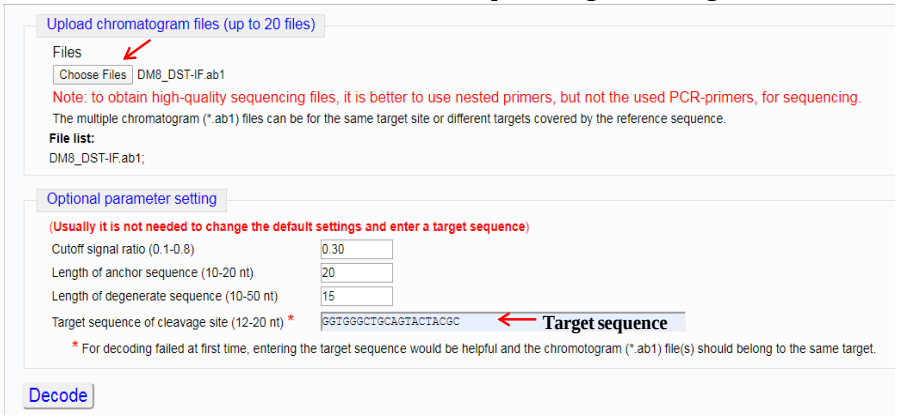
Mutant      ACTTCTTCTTCGGCGGCGGCGGCGGCGGCCGCGCATTCCAAGAAGGCAGGGTTATTC
Reference   ACCTCCTCCTCCGCCGCCGCCGCCGCCGCCGCGCAATCCAAGAAGGCAAGGTTAATC
            **  **  **  **  **  **  **  **  **  **  ********  ***********  *****  **
```

Decoding Targeted Mutations Using the DSDecodeM Tool

1. Either of two strands of the reference sequence can be used as a reference sequence. Hence, it is not necessary to convert the reverse complement of the reference sequence. However, it is important to check the quality of sequencing chromatogram before initiating a decoding task using DSDecodeM.
2. Load the submission page of DSDecodeM (http://skl.scau. edu.cn/dsdecode/). Input the intact wild-type reference sequence in the reference text area. The reference sequence must be identical to that of the parental individual for targeting, and completely cover the range of the sequencing file(s) for one or multiple target sites.

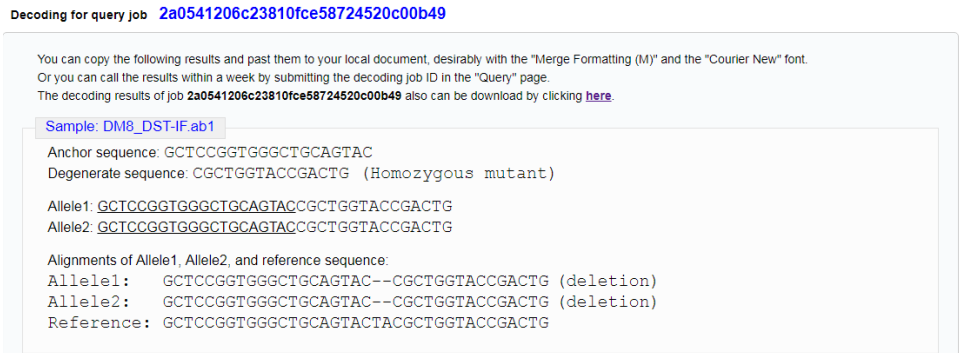


3. Upload the sequencing chromatogram file(s) in ab1 format (up to 20 files for one round decoding) from the same or different closely located targets. Reset some optional parameters if necessary, including the cutoff signal ratio (noise-peak/base-peak signal ratio), length of anchor and degenerate sequences, and inputting a target sequence. Generally it is not necessary to change the default settings or input the target sequence unless the decoding fails at first time. By adjusting these parameters, it is possible to exclude or reduce the interference from the sequencing noise signals

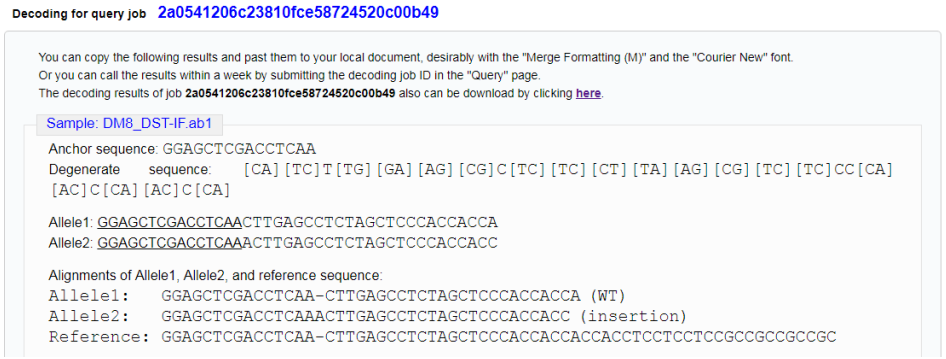


4. Click the "Decode" button to run the program. The result page of DSDecodeM displays all decoding result(s) of the uploaded sequencing file(s), including the AS and DS, and decoded allele 1 and allele 2 sequences, with their alignments with the reference sequence and indication of the mutation types. The decoding results can be downloaded into a txt format file or copied into a local document file.

Decoding of Target SgRNA1 resulted homozygous mutation



Decoding of Target SgRNA2 resulted heterozygous mutation



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Liu W, Xie X, Ma X et al (2015) DSDecode: a web-based tool for decoding of sequencing chromatograms for genotyping of targeted mutations. Mol Plant 8:1431–1433.

Chapter 16

Restriction enzyme digestion suppressed PCR assay to identify targeted gene mutation

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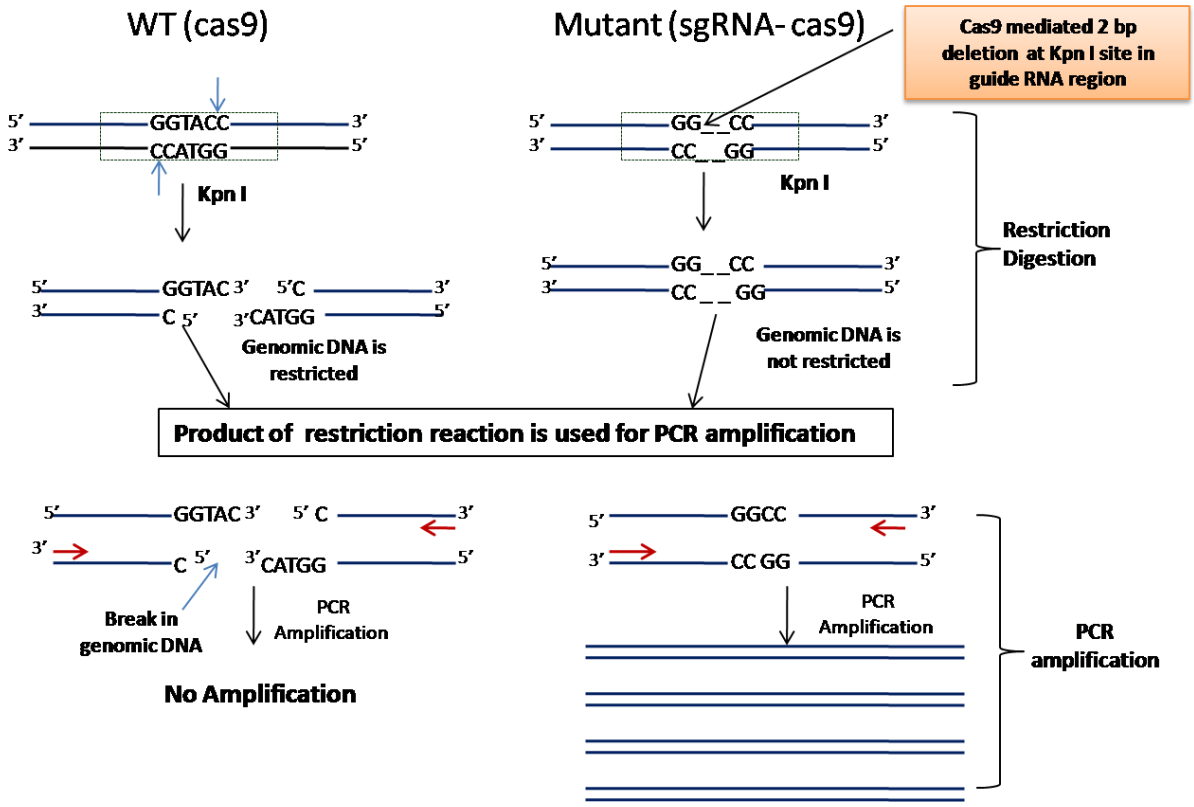
Principle

CRISPR- Cas9 based genome editing system enables editing of multiple genes under the guidance of small RNAs (sgRNA). Restriction and PCR based assays are important for fast and efficient mutation detection. Such methods allow first level screening of mutant line prior to sequence analysis. Targeted mutation at specific restriction enzyme sites can be detected by restriction enzyme digestion suppressed PCR assay. In this assay genomic DNA is isolated from lines transformed with binary vector containing only Cas9 (WT) and sgRNA-Cas9 (mutant). Digested genomic DNAs are used to amplify the targeted region using a pair of primers flanking the restriction site in gene of interest. Genomic DNA of wild-type plants have normal restriction site which will be digested by RE and PCR amplification in such lines will be dismissed or suppressed. In mutant lines mutation by CRISPR- Cas9 have destroyed the RE site thus DNA fragment is not digested and PCR amplification can be observed in such lines. This is clearly illustrated in the figure furnished below (Figure).

Materials required

- Nuclease free microcentrifuge tubes- 0.5 ml, 1.5 ml, 2 mL; Pipettes -10 µL, 200 µL, 1 ml; Pipette tips- 1 mL, 200 µL and 10 µL; Nuclease free PCR tubes
- Instruments: Dry bath, Water bath, PCR machine, Gel electrophoresis unit, Spin win, Gel documentation unit, Nanodrop.
- Reagents and components: Genomic DNA, Agrose, Restriction enzyme, RE buffer specific to RE, 10X Taq Buffer, dNTP Mix 2 mM each, Forward and Reverse primers, 25 mM $MgCl_2$, Taq Polymerase, Nuclease free water.



Detailed protocol

1. PCR amplification of wildtype and mutant lines were using nested primers surrounding the target SgRNA region. To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers and Taq Polymerase. Prepare sufficient master mix for the number of reactions plus one extra. Aliquot the master mix into individual PCR tubes and then add template DNA.

Sets for setting up PCR amplification reaction

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Place a thin-walled PCR tube on ice and add the following components for each 25 µL reaction:

S. No.	Components	Volume added	Final Concentration
1	Nuclease free water	To 25 µl	
2	10X Taq Buffer	2.5 µl	1 X
3	dNTP Mix 2 mM each	2.5 µl	0.2 mM
4	Forward primer	0.1-1 µM	0.1-1 µM

5	Reverse primer	0.1-1 µM	0.1-1 µM
6	25 mM MgCl2	2.5 µl	2.5 mM
Reaction mix is given a short spin at this point			
7	Taq Polymerase (*3unit/ µl)		1 U
8	Template DNA*	50-100 ng	2 µl

Gently spin down the content of PCR tubes. Incubate the reaction in PCR machine on mentioned thermal cycling condition.

S. No.	Steps	Temperature (°C)	Time	Number of cycles
1	Initial denaturation	95	1-3 min	
2	Denaturation	95	30 s	30-35
3	Annealing	Tm (55-60^{0}C)	30 s	
4	Extension	72	1 min/kb	
5	Final Extension	72	5-15 min	
6	Store	10		

2. Analyze PCR product by electrophoresis in 1.5-2% agrose gel.
   - Preprae Agarose gel, 1.5 % (w/v) in 1x TAE buffer. EtBr dye is added (2 µL per 100 mL gel) and mixed by swirling. The gel is casted in appropriate tray with comb.
   - Gel electrophoresis is performed in 1x TAE running buffer.
   - Add 6x loading dye in PCR product and load samples in to wells. Gel run is performed at 5 volts/cm. After 30 minutes, image is captured in gel documentation system.
3. Digestion of amplified target DNA with RE whose recognition sequence contains restriction enzyme site near the gRNA seed sequence region of target cleavge site of Cas9

I. Setting up of restriction enzyme digestion reaction- Take 0.5ml or 1.5 ml microcentrifuge tubes

S. No.	Components	Volume	Example
1	Nuclease free water	* To 50 µl	39 µl
2	RE Buffer (10 X)	5 µl (1 X)	5 µl
3	DNA	500 ng –1 µg	4 µl
Reaction mix is given a short spin at this point			
4	Restriction Enzyme (10U/ µl)	0.5-2 µl	2 µl
	Total	50 µl	50 µl

II. Incubate the reaction mixture at 37 ^{o}C (depends on R.E) for 2-3hrs in dry bath/ water bath.
III. Heat inactivate the restriction reaction by incubating at 65°C for 20 minutes

Precautions

- Gently vortex the buffer after thawing it properly.
- All components should be added in above mentioned sequence.
- After adding restriction enzyme avoid spinning of reaction mix.
- Restriction enzymes should be mixed properly by gentle tapping.
- Buffer of restriction reaction varies with the restriction enzyme and company. User should go through the user guideline of the company for selecting buffer and setting up the reaction.

Note: In RE-PCR Assay we can first do PCR amplification of fragment containing guide RNA sequence followed by its purification. Then, purified PCR product is digested by restriction enzyme specific for guide RNA (as seen in the figure below).

Example of PCR-RE Assay

PCR-RE is one of the best methods to confirm CRISPR/Cas9 genome edited plants as well as the zygosity of the mutant plants. Here we have shown one example to test mutation in CRISPR-Cas9 genome edited plants. Guide RNA's corresponding to target sites of *OsFTA* locus are designed for targeted mutation of this gene in rice. Primers were designed surrounding target region and amplified then digested with PflMI restriction enzyme. Digestion of amplified PCR fragments resulted two fragments if the target sequence is not having any mutation. If the target sequence was mutated then no digetion was occurred.

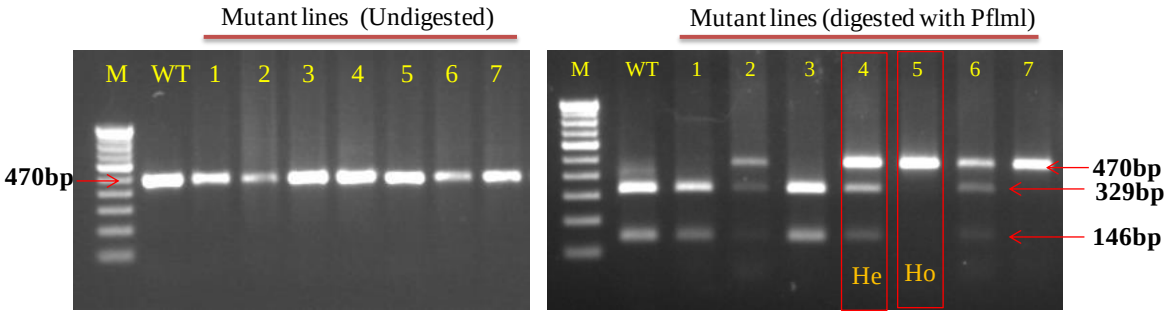
The schematic results were obtained given below

>Sequence

CAGCTGCTTGAGGAAGACGTCTTCAATAATTCAGCTTGGAATCAGGTCAGTT
GATATGCTTGTTTGGCTTTAAAATTTCTAGTTTATTATACAATTTCACTTTT
AATTCGTCCCATCTTGAATAGTAGTCTGAAAATCCATATGCCGGTTCATGAT
TTATTAACTAAATTCATTGATTCTTTCTCTTCTTGCTTTACGTGGTATTTTG
TCCTACTACTGTCTCCTTTCTGACTTTTTTTGGCTGATTAACTAAGATCAAA
TGATTACACTGAAGTATGTTATGTTTGTTTTGCAACAGAGATACCTTGTAAT
A**ACAAGTTCACCACTTCTTGG**AGGCCTTGCAGCAATGCGTGACTCGGAAGTG
GATTACACAGTTGGGGCTATTCTGGCTAACCCTCAGAATGAAAGCCCCTGGA
GATACCTCAAAGGCCTGTACAAGGGTGAAAATA**ACTTGCTGATGGCTGATGA
GC**

PflMI

Target SgRNA seqence **ACAAGTTCACCACTTCTTGG**



Ho- Homozygous, He-Heterozygous

References

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Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J. L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nature biotechnology, 32(9), 947.

Xie, K., & Yang, Y. (2013). RNA-guided genome editing in plants using a CRISPR–Cas system. Molecular plant, 6(6), 1975-1983.

Chapter 17

Detection and validation of InDels in genome edited plants by PCR

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Back ground

In PCR-based method, targets were amplified using two pairs of primers for each target locus and visualized on gel electrophoresis, Both methods can accurately define indel sizes dow-n to ± 1 bp, and are amenable for high throughput analysis, therefore, will significantly facilitate the identification of indel mutants generated by CRISPR/Cas9 for further functional analysis and breeding in crop plants. Small variations of nucleotides may cause loss of function through frame-shift mutation cannot be detected by conventional agarose gel electrophoresis.

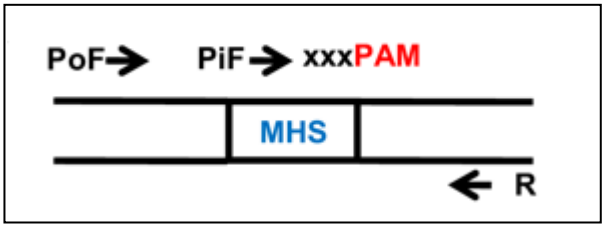
CRISPR/Cas9 induced mutation is predictable, because most of indels occur at the double strand breaks (DSBs) sites, namely mutational hot spot regions (MHS), generally within 4 base pairs upstream of the proto-spacer adjacent motif (PAM) (5′–3′). If the 3′ end of primers are designed to exactly cover these MHSs, theoretically, the CRISPR/Cas9 induced indels could be detected, because mismatches in the 3′ end of the primer will greatly reduce or completely loss the PCR amplification efficiency.

Materials required

Wildtype and Genome edited mutant lines
Genomic DNA extraction

Method

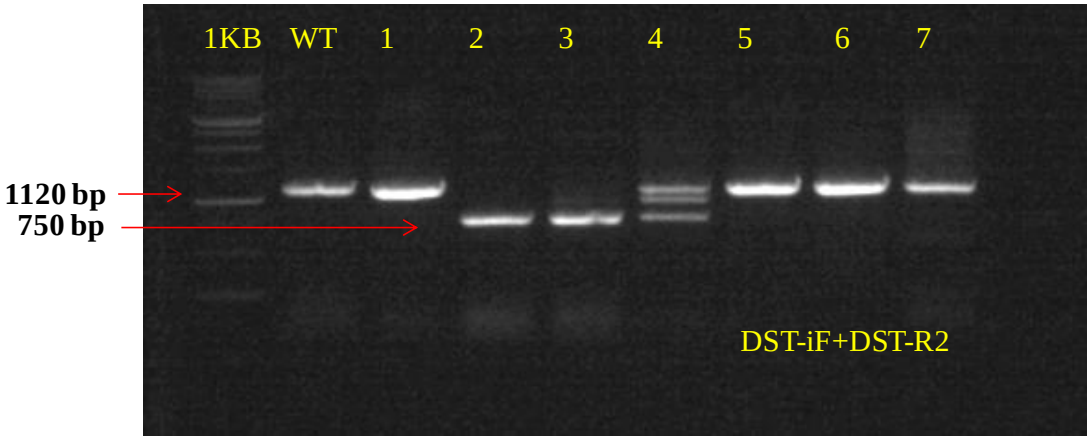
For PCR-based methods, the outer pair of primers were designed to prime outside the (Mutation hot spot region) MHS (PoF/R), and the inner pair of primers were designed to flank the MHS (PiF/R) at the 3´ most nucleotide of the MHS.

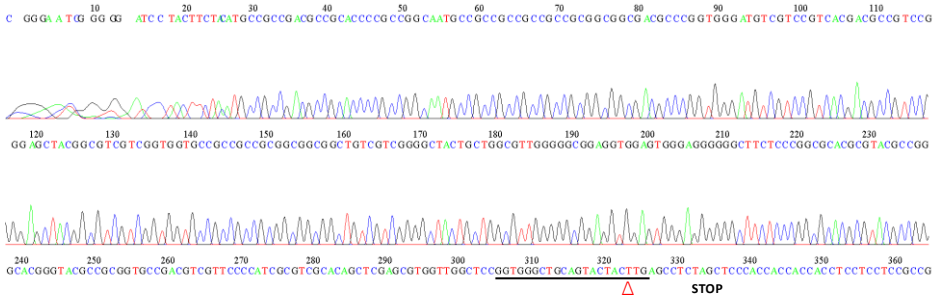


Multiple PCR was performed using a pair of inner (PiF/R) and outer (PoF/R) primers, and the resulting PCR products were analysed on 1.2 - 2% agarose gel.

Results

For PCR-based methods, the nested primers surrounding the target region were designed. PCR was performed and the resulting PCR products were analyzed on 1.2% agarose gel. For example OsX target gene different types mutant allelic variants were amplified and confirmed on 1.2% agarose gel electrophoresis. analysis. In the below figure mutant plant2 and 3 has deleted 366bp fradment and resultant was homozygous indel mutants were further proved by Sanger sequencing.






```
WT_DST-F.ab1        TTGGCTCCGGTGGGCTGCAGTACTACGCTGGTACCGACTGCGGCGCGGCGGCGGCGGGTG  359
D8-5_DST-F.ab1      TTGGCTCCGGTGGGCTGCAGTACTACΔTTGAGCCTCTAGCTCCCACCACCACCACCTCCTC  357
D8-6_DST-F.ab1      TTGGCTCCGGTGGGCTGCAGTACTACΔTTGAGCCTCTAGCTCCCACCACCACCACCTCCTC  360
                    **************************    *  * *  *  * * * * *
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References

Biswas, S., Li, R., Yuan, Z. *et al.* Development of methods for effective identification of CRISPR/Cas9-induced indels in rice Plant Cell Rep (2019) 38: 503. https://doi.org/10.1007/s00299-019-02392-3.

Glossary

Cas - CRISPR associated genes encodes for diverse set of cas proteins which are directly involved in CRISPR mechanism of action. CRISPR associated proteins binds to crRNA:tracrRNA complex to degrade foreign genetic material entering the cell. In genetic engineering, cas proteins forms a complex with gRNA to identify and cleave the target nucleic acid at a specific location.

Cas9 - Refers to the protein derived from the CRISPR-Cas bacterial immune system which has been adopted for genetic engineering. Cas9 uses gRNA to find a complementary DNA sequence. Cas9 cuts both strands once the target DNA is recognized. 3 bp upstream to the Protospacer Adjacent Motif, cas9 cleaves the target genomic loci. Cas9 derived from *Streptococcus pyogenes*, which has a PAM of 5'-NGG-3' (where N is any base), is the most commonly used nuclease. Both crRNA and tracrRNA or gRNA (fusion of crRNA and tracrRNA) is required correct binding and functioning of cas9 protein.

Cleave - Cleave, commonly refers to the splitting apart or cutting of DNA, RNA, or protein. In genome engineering, nuclease can be utilised to cleave the target DNA for gene editing.

Complementary - Nucleotide sequences which can form a double stranded structure by forming hydrogen bonds with the matching base pairs wherein, G is complementary to C and A is complementary to T (in case of DNA)/U (for RNA)

Cpf1 (Cas12) - A protein derived from the CRISPR-Cas bacterial immune system. After identification of the target DNA, Cpf1 cuts both strands of the helix. Cpf1, generates 4 or 5 sticky ends, with the double stranded DNA break, in contrast to the blunt end created with cas9.

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats. Genetic loci which constitutes an array of DNA repeats interspersed by short variable sequences called spacers, wherein the repeat sequences are palindromic in nature. The encoded CRISPR transcript is processed into small interfering RNAs. In genome editing, CRISPR system is utilised to create breaks in the target nucleic fragments.

CRISPR-Cas: Refers to the bacterial immune system comprising of a CRISPR repeat-spacer array and cas genes. Three CRISPR-cas systems are well defined, namely Type-I, Type-II, and Type-III, judged by the sequences and content of their constituents, particularly, cas genes.

CRISPR RNA (crRNA) - The host cell generates crRNA molecules as part of the bacterial immunity, each containing one spacer element which is complementary to a specific region of the invading viral genome. crRNAs help guide the CRISPR proteins to locate and subsequently destroy the matching invader sequences to cleave the target sites.In type II (Cas9) systems

crRNA interacts with tracrRNA to form a complex, which further associates with the Cas9 protein to form the CRISPR-cas9 complex.

CRISPRa and CRISPRi - CRISPRa stands for CRISPR activation. CRISPRi stands for CRISPR inhibition or interference. Both the methods are used in vitro for changing or fine tuning of the genome expression.

Double-stranded break (DSB) - Double strand break may be generated as a result of exposure to chemical, radiation or exogenous agents or also through the process of endogenous processes. It refers to the break in the double strand of the DNA where the strands of the helix are cleaved or severed.

Expression - A product being made from a gene; RNA or protein. After a gene is switched on, cellular machines "express" the gene by transcribing the DNA into RNA or translating RNA into amino acids to form proteins.

Gene therapy - Delivering corrected DNA to mammalian cells as part of the medical treatment. Gene therapy in plants classically, refers to stable integration of change to the genetic content of the somatic cells, followed by regeneration of plants.

Genetically modified organism (GMO) - Intentional genetic alterations to the DNA of certain organisms like microbes, plants and animals using specific scientific tools and technology.

Genome - Refers to the entire DNA content of an organism. The genome essentially contains set of instructions in the form of coding and non coding DNA to direct the development of individual parts and subsequent smooth running of functions.

Genome editing - Can be used to remove, change, or add DNA.

Genome Engineering - Genome engineering is the process of making deliberate specific changes to the genome of a cellular organism.

Guide RNA (gRNA) - Refers to a two-piece molecule formed by the fusion of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). Cas9 binds with gRNA sequence to detect specific PAM sequence of the target genome. tracrRNA functions as the guide of cas9, while the crRNA sequence directs the complex to the target matching DNA sequence. Advancement in genome editing has led to the development of single guide RNA to make engineering less complicated.

Homology-directed repair (HDR) - requires the presence of donor DNA to function as the template so as to patch the double stranded breaks in the DNA. For the donor template to be incorporated, it must contain similar sequences to the broken ends of the DNA. In genome engineering, donor DNA are designed to be inserted at DSB site, thus, allowing researchers to replace a disease-causing gene or replacing with the target gene.

HnH Domain - Part of the Cas9 nuclease, HnH domain contains two antiparallel beta sheets with two alpha helices present on each side.

Indel - An abbreviation used for insertion or deletion. It refers to the random removal or insertion of nucleotides at the target site. Indels occur when DNA is broken and is repaired by the cell by a process called non-homologous end joining (NHEJ). NHEJ is error-prone and can thus, result in generation of non functioning genes.

Interference - A process reliant on complementarity binding of the crRNA and the target nucleotide sequence, interference refers to the targeting of the DNA/RNA sequence of the invaders by the cas proteins.

Knock-Out - Generation of knock out lines, refer to the insertion or deletion of short heterogenous segments at the site of disruption resulting in knocking out of the target gene or nucleotide sequence.

Knock-In - Homology repair leads to insertion of DNA template at the site of disruption which can be utilised to create knock in lines with target genes in genome editing experiments. Leader - Refers to the AT-rich sequence present upstream to the first CRISPR repeat. It functions as a promoter for regulated transcription of the repeat-spacer array.

Mutation - Mutation refers to the change of one nucleotide base to the other. While certain mutations have no consequence at allie, are silent, certain mutations can directly lead to the development of disease by causing hindrance to the gene expression. Mutations can be caused by various DNA-damaging agents such as UV light or it may arise endogenously by formation of errors when DNA is copied by cellular enzymes. They can also be instigated deliberately via genome engineering methods.

Non-homologous end joining (NHEJ) - A way for a cell to repair a break in its DNA by attaching the free DNA ends. This pathway is error-prone, and often results in the random addition or removal of nucleotides around the site of the DNA break, causing insertions or deletions in the genetic code.

Nick - Formation of a gap in the backbone of a single strand of the DNA without DNA separation. CRISPR cas9 functions as a genome engineering tool to create a nick in the target strand.

Nuclease -Refers to enzyme which are responsible for cleaving of DNA or RNA by breaking the phosphodiester bonds between nitrogenous bases. Breaking of a single strand can generate a nick and breaking of both strands results in a double-strand break. Exonuclease cuts from the end of the DNA strand whereas an endonuclease cleaves from the centre of the DNA or RNA. For example, Cas9 are endonucleases used in genome engineering.

Off-Target Mutation - Off-target Mutation refers to the probability of the guide RNA binding to off-target site in place of the target site due to the low specificity of cas9 enzymes and similarity between the two sites.

Pre-crRNA - pre-CRISPR RNA. It is the full length transcript transcribed by the CRISPR spacer-repeat array. Pre-crRNA serves as the precursor for crRNA biogenesis.

Protospacer - It is the precursor sequence of CRISPR spacers present in the DNA of invasive elements which will be taken up by the CRISPR-Cas immune system. Subsequently, it is also targeted by crRNA as part of the CRISPR-cas9 immunity.

Protospacer adjacent motif (PAM) - A 2-5nt sequence present downstream to the DNA target sequence for Cas9 to bind and further cut. Downstream presence of Protospacer Adjacent Motif is essential to binding of cas proteins. In bacteria, PAM is present on the foreign nucleic material of the invading virus, due to which the bacterial immunity system is able to distinguish between self and foreign and prevent cleavage of host.

Repeats - short sequence, separated by the spacer sequences, part of the CRISPR array. A subset of all repeat types are palindromic in nature. CRISPR repeats are imperative for crRNA maturation, and forms a complex with cas genes to form a functional CRISPR-Cas system.

Ribonucleoprotein complex (RNP) - Refers to an assembly containing both protein and RNA. In genome engineering, it is often used to describe gRNA-cas9 complex, which form an active enzyme. For genome editing in cells, Cas9 can be delivered as an assembled RNP to cells, or can be delivered as DNA or RNA which will contain the general instructions for formation of RNA or protein components.

RuvC - It is an endonuclease domain of cas proteins and plays a critical role for cleavage of the target site. It is responsible for initiation of cutting of DNA which is not complementary to the guide RNA.

Seed sequence - It is a short stretch of nucleotides sequence, usually 7-9 nt in length, within the crRNA. It requires perfect base pairing between the complementary sequence of crRNA and the target nucleotide sequences.

Single-guide RNA (sgRNA) - A simplified version of the two piece guide RNA, engineered together to form a single complex. sgRNA provides less complication and facilitates direct binding of the cas9 protein to cleave the DNA sequence for genome editing. It is a fusion of crRNA and tracrRNA which is utilised in CRISPR/Cas9 systems.

Strand - Refers to a string of chained nucleotides; Two strands of DNA can zip up to form a helix due to the complementarity of the bases.

Transcription activator-like effector nuclease (TALEN) - It is a genetic engineering tool formed by the fusing of DNA binding domain with a DNA cleavage domain and is used to restrict the target DNA in genome engineering.

Trans-activating CRISPR RNA (tracrRNA) - Abbreviation for trans-activating CRISPR RNA, it is pronounced as "tracer RNA." The tracrRNA base pairs with crRNA to form a guide RNA (gRNA). tracr-RNA is complementary to the pre-crRNA and forms a duplex which is then subsequently cleaved to form crRNA-tracrRNA complex.

Transcription - The process by which DNA information is transcribed to a strand of RNA with the aid of enzymes like DNA polymerase.

Translation - The process by which a chain of amino acids are formed from the information coded in the RNA. It is performed with the aid of multiple proteins and enzymes (for eg, ribosomes)

Zinc-finger nuclease (ZFN) - Range of engineered DNA binding proteins which are facilitate editing of target genomic loci on the basis of protein guided genomic cleavage.