

Transgene (DNA)-Free Genome Editing



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Generation of Biotechnologies

Refers to different eras in biotechnological techniques & applications

First Generation of Biotechnology

Initial phase in the use of biotechnological techniques

Key aspects

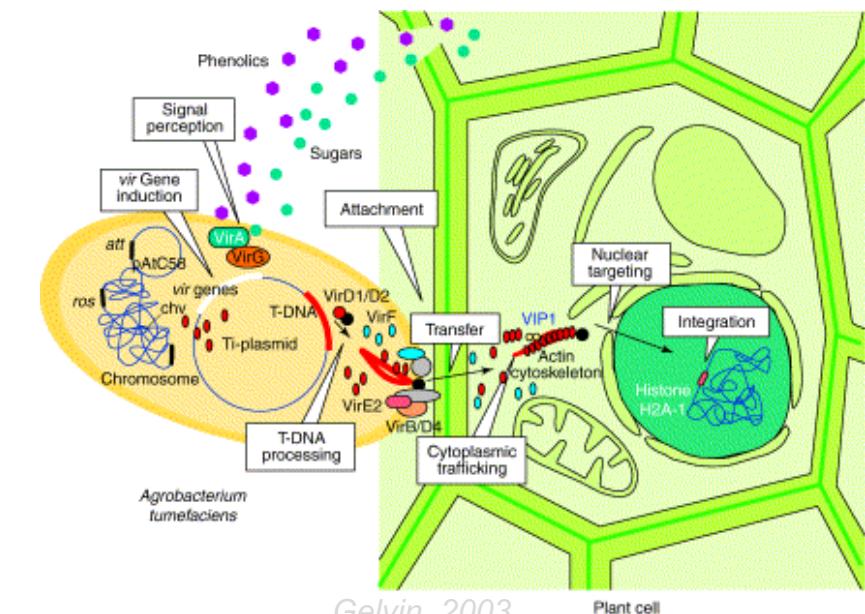
- ❖ Application of traditional breeding methods combined with emerging molecular biology techniques
- ❖ Development of Tissue Culture Techniques

Early Genetic Engineering

- Genetic Transformation
- Development of Transgenic Plants



www.plantcelltechnology.com



Second Generation of Biotechnology

Represents more sophisticated techniques and applications, particularly in genetic engineering and molecular biology.

Key Aspects

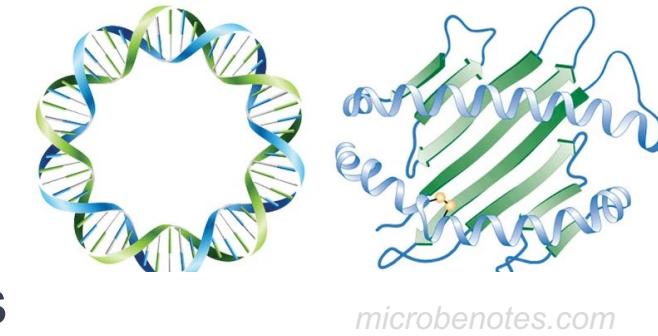
Advanced Genetic Engineering- Creation of genetically modified organisms (GMOs) with specific traits and more complex manipulations of DNA, like gene silencing & gene editing

Proteomics and Genomics- Study and manipulation of entire genomes and proteomes for understanding complex biological processes and finding novel traits to control diseases and pests

Bioinformatics and Computational Biology- Using computational tools to manage and analyze biological genomics and metabolomics data and understanding biology



Azevedo & Arruda, 2010



microbenotes.com



<https://cb.iiitd.ac.in/>

Third Generation of Biotechnology

Represents an even more advanced phase in the evolution of biotechnological applications, with cutting-edge techniques and innovative approaches.

Key aspects

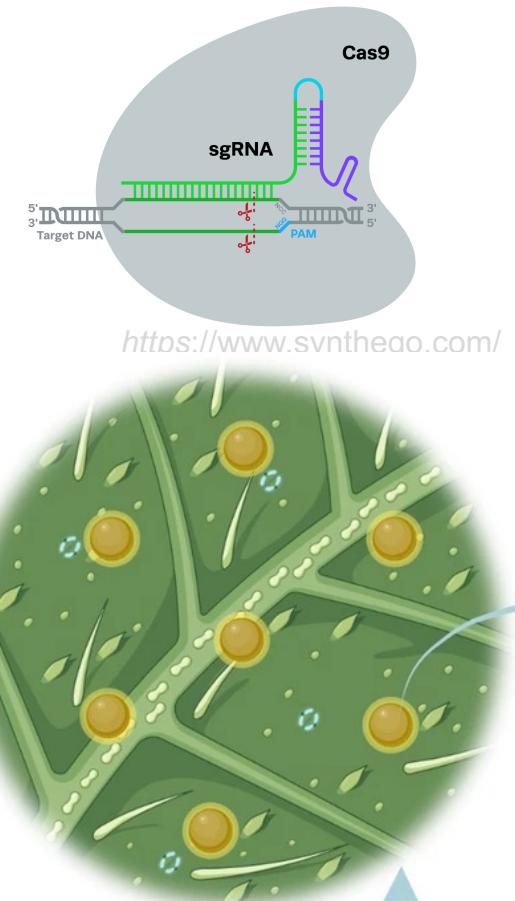
Advanced Gene Editing Technologies

CRISPR-Cas9 become more refined, allowing for targeted gene editing with higher accuracy and fewer off-target effects.

Biofortification

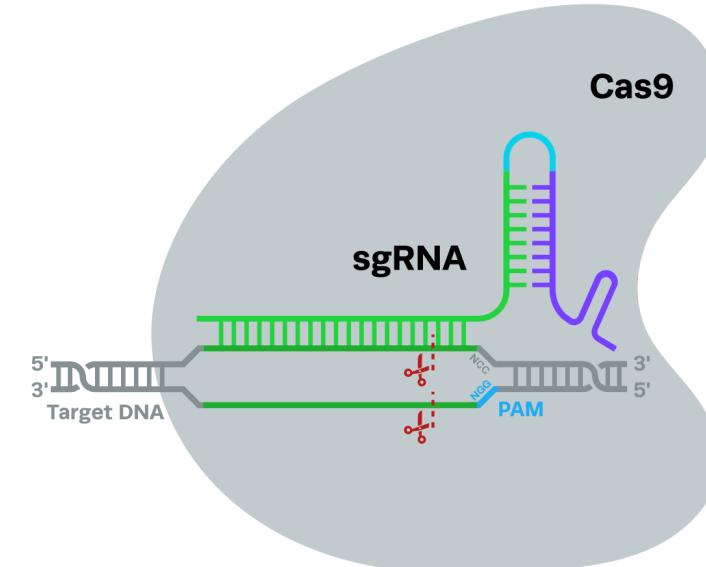
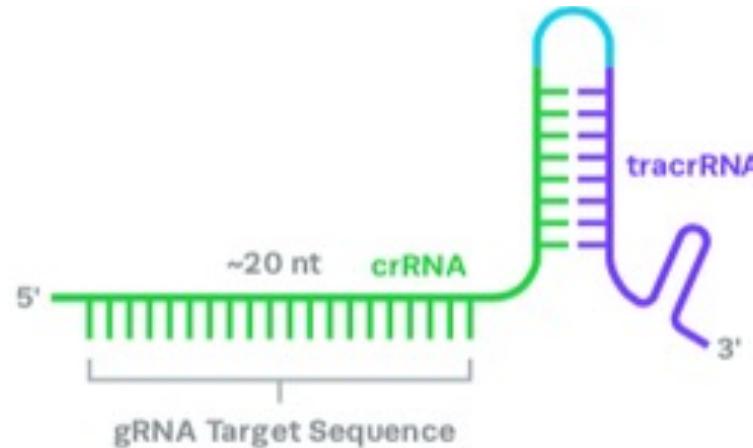
Metabolic Engineering

Use of NanoPhytoBiotechnology



New Breeding Techniques (NBTs)-Targeted Gene Editing

- ❖ Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein-9 (CRISPR-Cas9)
- ❖ Directing a guide RNA (gRNA) complementary to a defined target, together with a CRISPR-associated endonuclease, Cas9, to create double-stranded DNA cleavage
- ❖ gRNA: crispr RNA (crRNA), a 17-20 nucleotide sequence complementary to the target DNA, and a tracrRNA, which serves as a binding scaffold for the Cas nuclease



Where to start.....

Method

Crop?

Tissue culture?

Gene Information and gRNA Design?

Mutant screening?

Regulation?

Rapeseed

- ❖ Third-largest source of vegetable oil after soybean and palm oil
- ❖ The terms “rapeseed”, “canola”, “mustard” interchangeably used in different continents
- ❖ In Europe, it is known as rape and oilseed rape
- ❖ In India, use of rapeseed oil in lamps since 2000 BC
- ❖ In Canada, lubricant for war ships (1936)
- ❖ In Europe, making food and soap since 13th century
- ❖ Rapeseed (traditional name) - group of oilseed crops in the Brassicaceae family, and naturally contains high amounts of erucic acid (>45%) and glucosinolates
- ❖ ‘Canola’ refers to the edible oil crop - low erucic acid (less than 2%) and <30 µmol/g of dried defatted meal of glucosinolates -developed via traditional crossbreeding



Genome organization

- ❖ Rapeseed, *Brassica napus* subspecies, *napus*, is a large winter or spring annual oil crop
- ❖ Rapeseed is related to Arabidopsis, mustard, cabbage, broccoli, cauliflower and turnip
- ❖ Many important crops are polyploid, and rapeseed is one among them
- ❖ Polyploid plants are generated by evolutionary processes and/or crop domestication
- ❖ Doubling of genomes at least once in evolutionary history, resulting in polyploidy
- ❖ Autopolyploid- whole-genome duplication event
- ❖ Allopolyploid- interspecific or intergeneric hybridization event followed by chromosome duplication



Rapeseed



Nutrient Composition

Fats **40-45%**

MUFA **Oleic acid (ω-9)** 60-65% Stable

PUFA

linoleic acid (ω-6) 20%

α-linolenic acid (ω-3) 9-11%

1:1

SFA **7-8%**

Erucic acid **< 2%**

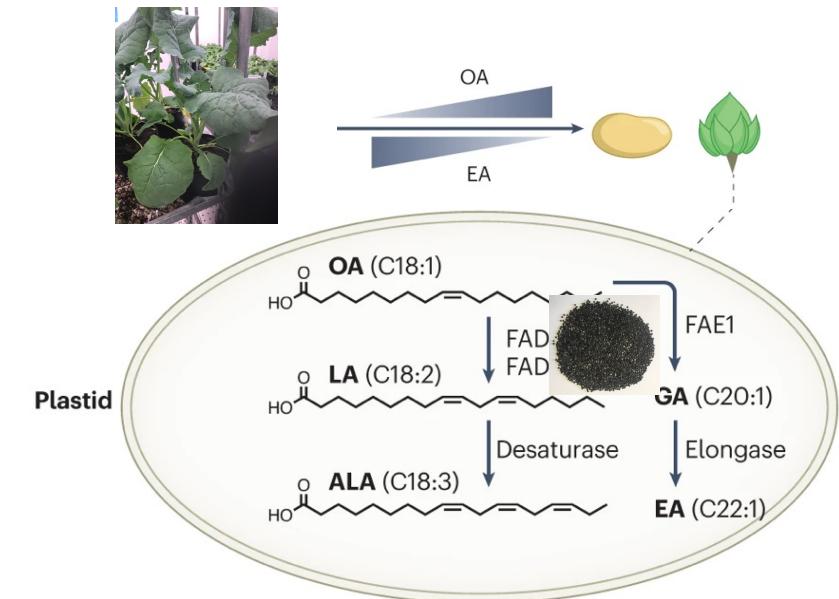
Protein ~ 20%

Fiber ~14%

Carbohydrates < 10-15%

Vitamins E & K

Minerals



Tuncel et al. 2023 *Nature Reviews Bioengineering*

Genetic Improvement in Rapeseed

- ❖ Rapeseed (AACC, $2n = 38$)
- ❖ Interspecific hybridization - *B. oleracea* (CC, $2n = 18$) and *B. rapa* (AA, $2n = 20$)

Major Quality issues/Challenges: Erucic acid and Glucosinolates

- ❖ 1970 -Intensive breeding programs
- ❖ Back-cross with high erucic acid line to high erucic acid cultivars for non-food purposes
- ❖ Back-cross with low erucic acid line and low glucosinolate line to develop low erucic-acid and glucosinolate cultivars



Agronomic traits and oil quality

By-products of oil industry- Oilseed cake or meal?

- ❖ Second-largest source of protein meal (heat and press)/cake (solvent)
- ❖ Source of protein, energy, carbohydrates and mineral contents



Challenges

- ❖ Highly complex and repetitive nature
- ❖ More copies of each gene that are identical to each other
- ❖ Chromosomal rearrangements and epigenetic shifts
- ❖ Activation of transposable elements,
- ❖ Neo- and subfunctionalization events after gene duplication
- ❖ Co-editing of multiple alleles/genes



Advantages

- Generation of a range of functionally differing phenotypes by partial loss of expression
- Complete loss-of-function may impair plant performance

Antinutrient factors (ANF) in rapeseed

Seedcake/meal

Protein: 35-45%

Rich in Tryp & Threonine

Good amounts of Lys, Met & Cys

Fiber: 10-12%

Fat: 3-5%



Photo: Anja Persson

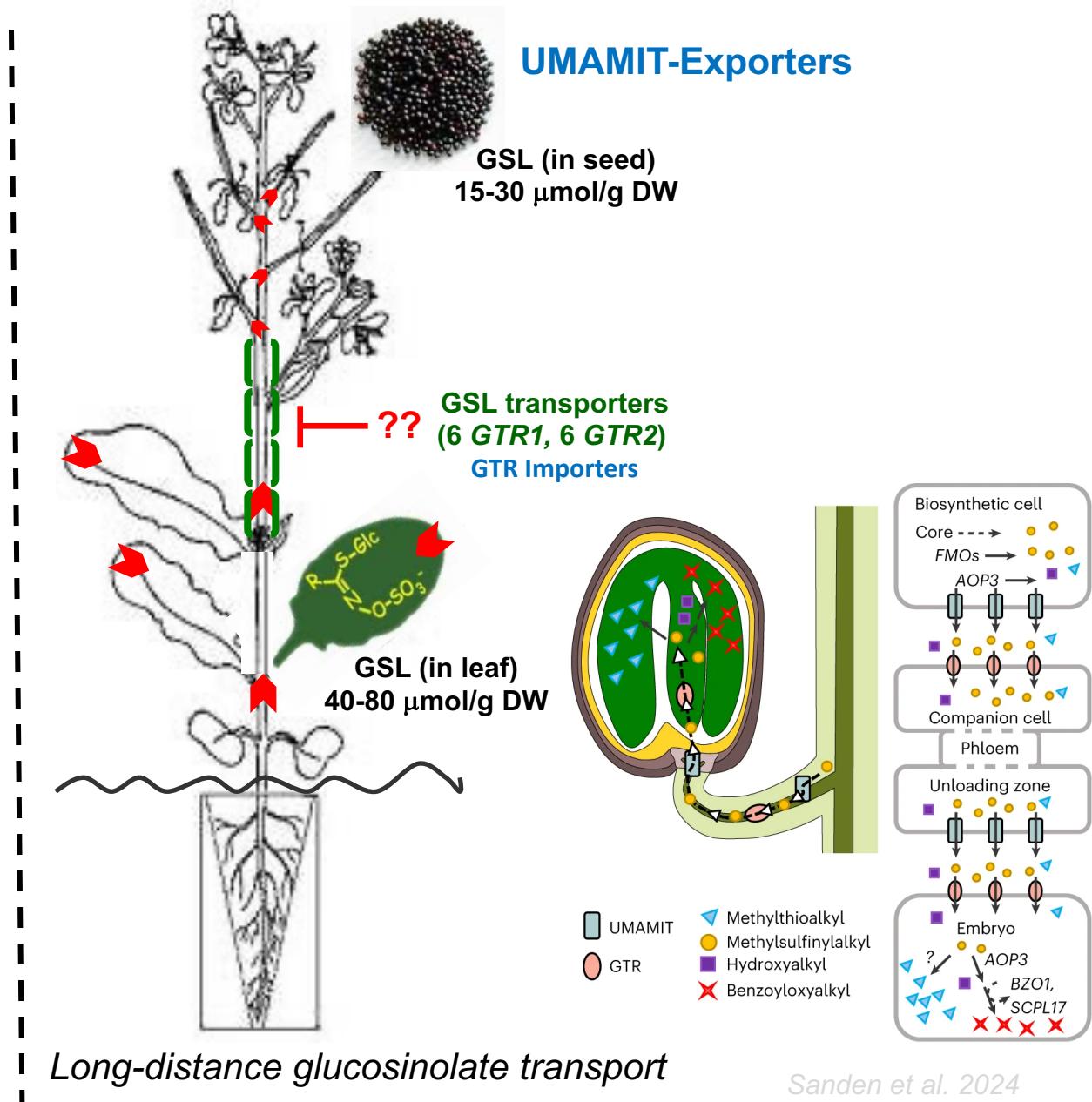
Anti-Nutritional Factors

1. Erucic Acid: Harmful to heart health

2. Glucosinolates: Sulfur-containing compounds-bitter-
interfere with thyroid function

3. Sinapine: A phenolic compound - bitter taste- binds
to minerals and digestive enzymes

4. Phytic Acid: Binds to minerals, iron and zinc



Glucosinolates

- ❖ Synthesized in vegetative tissues and transported to seeds which is mainly regulated by glucosinolate transporter (*GTR*) genes (*GTR1* and *GTR2*)
- ❖ Glucosinolates-sulfur- and/or nitrogenous secondary metabolites
- ❖ Glucosinolates-defense compounds against pests
- ❖ Antinutritional and negative physiological effects
- ❖ Pungent flavor in rapeseed is imparted by glucosinolates

Constraints in traditional breeding and MAS

- ❖ Multiple homoeologous copies (alleles) of each gene
- ❖ Challenges in mutation breeding
- ❖ Extensive backcrossing or introgression

Gene identification

Functionally characterized genes from *Arabidopsis*

tair

Home Help Contact About Us Subscribe Login Register Institution: Swedish University of Agricultural Sciences (subscribed)

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Locus: AT3G47960

What's new on this page Add a Comment

Representative Gene Model **AT3G47960.1**

Gene Model Type protein_coding

Other names: ATNPF2.10, GLUCOSINOLATE TRANSPORTER-1, GTR1, NPF2.10, NRT1/PTR FAMILY 2.10

Description Encodes a high-affinity, proton-dependent glucosinolate-specific transporter that is crucial for the transport of both methionine- and tryptophan-derived glucosinolates to seeds.

Center on AT3G47960 | Full-screen view

17,698,750 17,700,000

Araport11 - Protein Coding Genes

AT3G47960.1 Major facilitator superfamily protein

Annotations category relationship type keyword

Arabidopsis database

GTR1 gene

Brassica napus databases

Introduction Databases Details

Brassica Genome



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Brassicaceae Database

- The **Brassicaceae Database (BRAD)** was previously a searchable Database of *Brassicaceae* genomic data hosted on the website "<http://brassicaidb.org>". For the policy and safety reasons, we moved it to the new website "<http://brassicaidb.cn>". It is now a new database which includes not only newly released genome sequences of *Brassicaceae* species, but also published genomic data of most other *Brassicaceae* species.
- In total, we provided services for 35 genomes or genome versions from 25 species (refer to [Species info](#)). The genomic data include mainly genome assemblies, predicted gene models and gene annotations.
- These data can be browsed in iBrowse or searched in BLAST. We continue offering the featured service of searching for syntenic genes, which are generated based on their synteny relationships to the genes in *Arabidopsis thaliana*.
- We will regularly update the database with newly released high-quality reference genomes.

News
BRAD V3.0 has been launched for trial. We welcome any feed back for improving the website.

Group Leader
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Website Developer
Haiwei Chen
E-mail: caas_iwv_chenhaiwei@163.com

Visitors

Pageviews: 12,921

 [Ensembl Plants Home](#)

 **Brassica napus (AST_PRJEB5043_v1)**

[HMMER](#) | [BLAST](#) | [BioMart](#) | [Tools](#) | [Downloads](#) | [Help & Docs](#) | [Blog](#)

 **Brassica napus Assembly and Gene Annotation**

  Centre National de Séquençage

Brassica napus Genome Browser

[Genoscope](#) [Home](#) [Browser](#) [Blat](#) [Synteny](#) [Download](#)

Browse Data

chrA01

Search using a sequence name, gene name, locus, or other landmark. The wildcard character * is allowed.

Search: Display:

chrA01:6,320,000..6,340,999 region of chromosome chrA01 from 6,320,000 to 6,340,999
chrC01:11,338,000..11,372,999 region of chromosome chrC01 from 11,338,000 to 11,372,999
BnaA01g00030D Gene prediction BnaA01g00030D


B. napus Browser

NCBI databases for *B. napus*

Search NCBI

brassica napus

Search

Results found in 22 databases

TAXONOMY

Brassica napus

Rape (*Brassica napus*) is a species of eudicot in the family *Brassicaceae* (mustard family).

Taxonomy ID: 3708

Was this helpful?  

Genomes
Browse all *Brassica napus* genomes

Literature

Bookshelf	86
MeSH	39
NLM Catalog	10
PubMed	7,694
PubMed Central	31,076

Genes

Gene	149,762
GEO DataSets	3,448
GEO Profiles	0

Proteins

Conserved Domains	8
Identical Protein Groups	355,912
Protein	775,029
Protein Family Models	7
Structure	40

Genomes

Assembly / Genome	NCBI Datasets	16
BioCollections	0	

Clinical

ClinicalTrials.gov	0
ClinVar	0

PubChem

BioAssays	119
Compounds	2

NCBI database for *B. napus*

Descriptions Graphic Summary Alignments Taxonomy

Sequences producing significant alignments Download [Select columns](#) Show 10 ?

select all 10 sequences selected [GenBank](#) [Graphics](#) [Distance tree of results](#) [MSA Viewer](#)

	Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106397267), mRNA	Brassica napus	rape	3708	1912	1912	90%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106410496), misc_RNA	Brassica napus	rape	3708	1912	1912	90%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10 (LOC106445255), mRNA	Brassica napus	rape	3708	1906	1906	90%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106408997), mRNA	Brassica napus	rape	3708	1890	1890	90%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC111202315), mRNA	Brassica napus	rape	3708	1873	1873	91%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106414122), mRNA	Brassica napus	rape	3708	1845	1845	91%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106384518), mRNA	Brassica napus	rape	3708	1480	1480	71%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.11-like (LOC106366160), transcript variant X2, misc_RNA	Brassica napus	rape	3708	1040	1040	87%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.11-like (LOC106366160), transcript variant X1, mRNA	Brassica napus	rape	3708	1040	1040	87%
<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.11-like (LOC106366161), mRNA	Brassica napus	rape	3708	1035	1035	87%

[Download](#) [GenBank](#) [Graphics](#) ▾ Next ▲ Previous [Descriptions](#)

PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106397267), mRNA
 Sequence ID: [XM_013837850.2](#) Length: 2254 Number of Matches: 1

Range 1: 370 to 2106 [GenBank](#) [Graphics](#) ▾ Next Match ▲ Previous Match

Score 1912 bits(1035)	Expect 0.0	Identities 1509/1743(87%)	Gaps 12/1743(0%)	Strand Plus/Plus
Query 147	GGTCGATTCTTCGAGGAAGAGCAGAGAAAAATCGTTTATAGAGGATGGAAAGTCATGCC			206
Sbjct 370	GCGTCAATTGAAAGACGAGCAGAAAAAGCTCGTTATAGAGGCTGGAAAGTCATGCC			429
Query 207	TTTTATCATTGTAATGAGACATTGAGAACGCTGGGATCATAGGGACATTATCAAACCT			266
Sbjct 430	CTTATCATTGTAATGAGACATTGAGAACGCTGGGACACTATCAAACCT			489
Query 267	TCTTGTGTACCTAACCTCTGTATTCAACCTTAAAGAGCTACACAGCTGCAACTATCATCAA			326
Sbjct 490	TCTGGTGACCTAACCTCTGATTCAACCTCAAGAGCTGGTACAGCTGCAACCATCATCAA			549
Query 327	TGCCTTGTGGTACAATCAATTTCGGGACTTTTATTGCTGCCTTCTTGCACACTTA			386
Sbjct 550	CGCTTCAGTGGCACTATCAACCTCGGCACCTCCCGCTGCTTCTGGACACTTA			609
Query 387	CTTTGGTGGTACAAGACTCTCAGTGTGCGCTGTCATGGCTTCTGGGATCGTTGT			446
Sbjct 610	CTTGGTGGTACAAGACTCTCTGTCATGGCTGCTTCTGGGATCGCTTGT			669
Query 447	GATACTACTTACTGCTGCAATTCCGTCGTTGCACCCCGTTGCTTGGAA-ACAAAAATCT			505
Sbjct 670	GATACTACTGACGGCTGCAATTCCAGGATTGCAACCCATTCTTGTGGAACACAAAGT-			728

Related Information
[Gene](#) - associated gene details
[Genome Data Viewer](#) - aligned genomic context

B. napus pan-genomic database

Rapeseed Genome Facts

BnPIR Rape Genome Annotations, release v0:

Accessions	ZS11	Westar	No2127	Zheyou7	Gangan	Shengli	Tapidor	Quinta
Gene Number	100,919	97,514	95,385	96,209	96,843	94,586	96,117	95,492



Basic Local Alignment Search Tool

BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases (*B. rapa*, *B. oleracea*, and eight *B. napus* accessions) and calculates the statistical significance.

BLAST program

Organism:

Database

Query sequence

BLASTN: NT query, NT db

ozs11 westar tapidor no2127 darmor Bra Bol

zs11_CDS(-UTR-intron)(NT)

ATGAAAGAGCAGAGTCATCCACAGCCATAGAGAGAGAAGAGATAAGCATAATTACACAGATTGAAACAACTGGAG
AGAAAAGCCCTTGTGAGGACAATCTAGGGATCACAACCCCTACTCTCCGCGTGGCGGTGGTTCTGATTC
GGTCGATTCATTGAAAGCAGCGCAGAAAAGCTCGTTATAGGGCTGGAAAGTCATGCCCTTATCATTGGTAAT
GAGACATTGAGAAGATAGGGATCATTGGGACACTATCAAACCTCTGGTGTACCTAACCTCAGTATTCAACCTCA
AGAGTGTACAGCTGCAACCATCATCACCGCCCTCGATGGCACTATCAACTTCCGCATCTTCCTGCTGCTTCC
TCTGCGACACTTACTTGGCTGCTCAAGACTCTCTGCTGCTGCTCATGCCGCTTTCCTGGATCCTGTTGTA
TACTACTGACGGCTGCAGTTCAGGATTCAGGACCCATTCTTGTGAAACAAAGTCTTGCAAGGCCAAGGCCAAGC

Descriptions

Description	Max score	Total score	Query cover	E value	Ident
BnaA06702320900N0	3519	3519	100%	0	100%
BnaA06702340400N0	3342	3342	100%	0	98%
BnaA06701655000N0	2243	2243	93%	0	93%
BnaA06703543600N0	2237	2237	96%	0	89%
BnaA01701920000N0	2097	2097	95%	0	88%
BnaA06702212000N0	2075	2075	95%	0	87%
BnaC03704626800N0	1129	1129	88%	0	79%
BnaA06702817000N0	1118	1118	88%	0	79%
BnaC027044843800N0	1111	1111	89%	0	79%
BnaA02703923000N0	1091	1091	94%	0	78%
BnaC09700423000N0	1067	1067	88%	0	78%

Alignments

> BnaA06T0259000NO locus=nc02127v0 A06:32588651:32591193:+ Length: 1905 Number of Matches: 1

Range 1: 1 to 1905

	Score 3519 bits (1905)	Expect 0	Identities 1905/1905 (100%)	Gaps 0/1905 (0%)	Strand Plus/Plus
Query 1	ATGAAGAGCAGAGTCATCACCACGATAGAGAGAGAGATAAGCATAATTACACAG	60		
Sbjct 1	ATGAAGAGCAGAGTCATCACCACGATAGAGAGAGAGATAAGCATAATTACACAG	60		
Query 61	ATTTGAAACATGGAGAGAACGCTTGTGTTGAGCACACTGAGGATCACAACTTCAC	120		
Sbjct 61	ATTTGAAACATGGAGAGAACGCTTGTGTTGAGCACACTGAGGATCACAACTTCAC	120		
Query 121	CCCTCCGTGATGGCGGTGTTCTGATTCGGTCGATTCTGGATTTGAGACGAGCAGAAAAAG	180		
Sbjct 121	CCCTCCGTGATGGCGGTGTTCTGATTCGGTCGATTCTGGATTTGAGACGAGCAGAAAAAG	180		
Query 181	CTCGTTATAGAGCTGGAAAGTCATGCCCTTATCATGGTAATGAGACATTGGAGAAG	240		
Sbjct 181	CTCGTTATAGAGCTGGAAAGTCATGCCCTTATCATGGTAATGAGACATTGGAGAAG	240		

Database Name	Source
<i>Brassica napus</i> pan-genome v3	
<i>Brassica napus</i> Westar genome	
<i>Brassica napus</i> Kale genome	
<i>Brassica napus</i> Mendel genome	
<i>Brassica napus</i> LT genome	
<i>Brassica napus</i> ZS11 genome FAFU	Chen et al. 2020
<i>Brassica napus</i> ZS11 genome HZAU	Song et al. 2020
<i>Brassica napus</i> ZS11 genome v201608	Sun et al. 2017
<i>Brassica napus</i> Darmor genome v4.1	Chalhoub et al. 2014
<i>Brassica napus</i> Darmor genome v10	Rousseau-Gueutin et al. 2020
<i>Brassica napus</i> Express617 genome v1	Lee et al. 2020
<i>Brassica napus</i> NY7 genome v2	Zou et al. 2019
<i>Brassica napus</i> ganganF73 genome	Song et al. 2020
<i>Brassica napus</i> no2127 genome	Song et al. 2020
<i>Brassica napus</i> quintaA genome	Song et al. 2020
<i>Brassica napus</i> shengli3 genome	Song et al. 2020
<i>Brassica napus</i> tapidor3 genome	Song et al. 2020
<i>Brassica napus</i> westar genome HZAU	Song et al. 2020
<i>Brassica napus</i> zheyou73 genome	Song et al. 2020

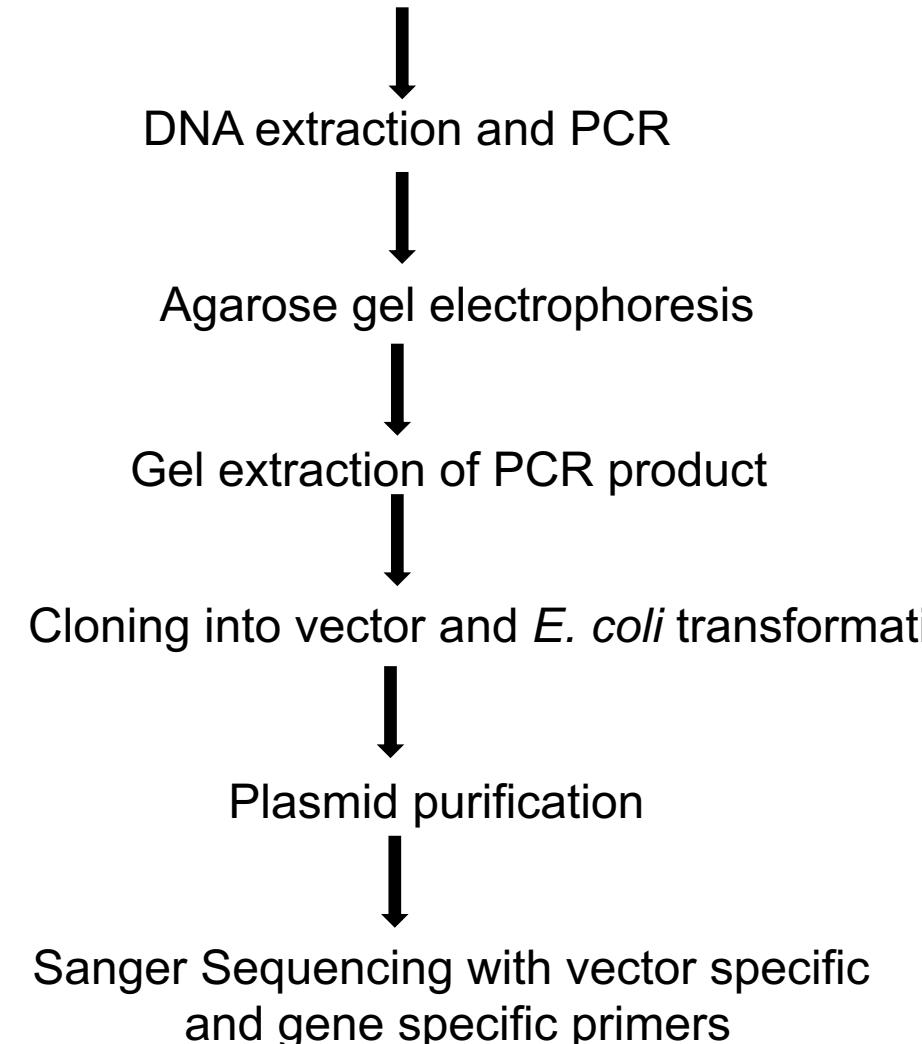
Whole Genome Sequencing

Bottlenecks in the gene identification

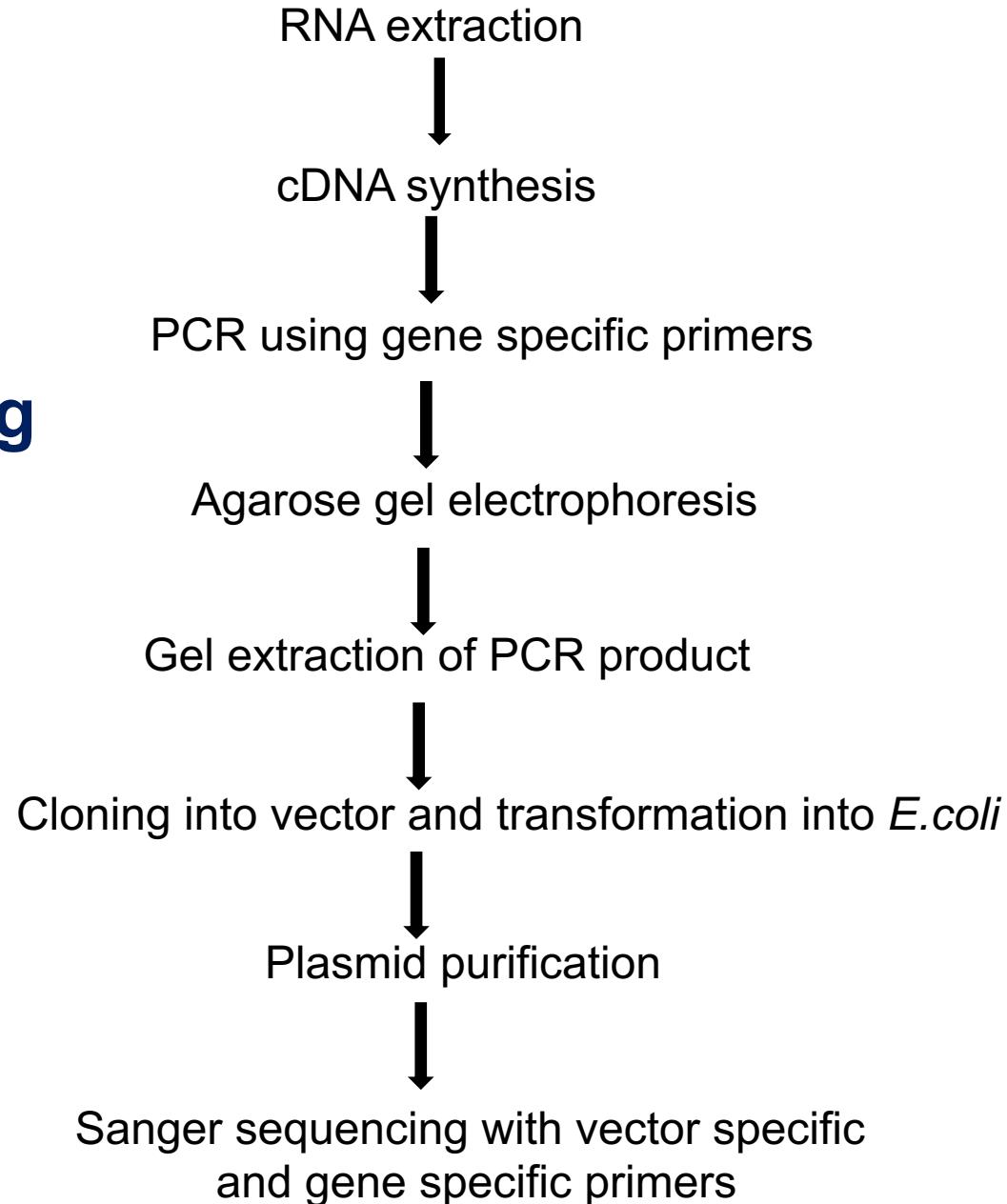
- ❖ DNA sequence of genes is needed for gene editing
- ❖ Chromosomal-level gene assemblies are needed
- ❖ Identical copies of genes in the same chromosome

General workflow for cloning of a target genes

Primer design based on untranslated regions/genes based on reference genome



General workflow for cloning of a target gene from cDNA



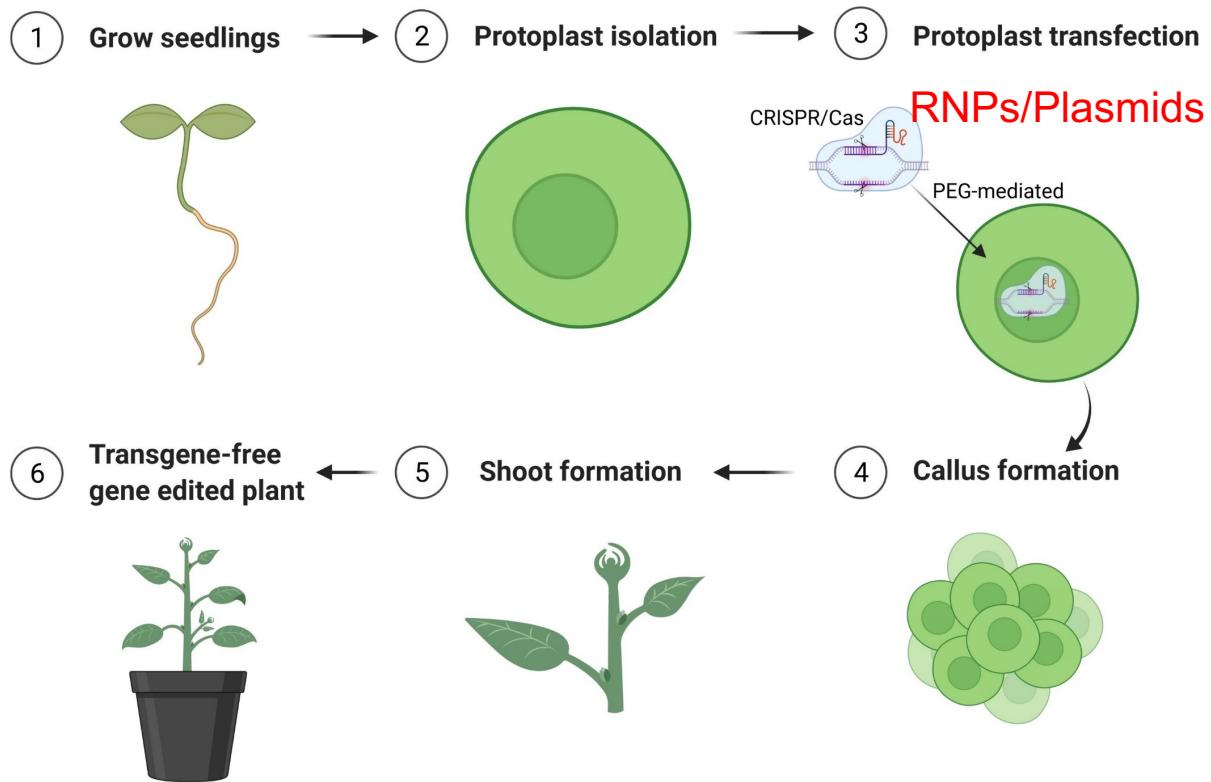
Nucleotide similarity of *GTR1* genes in *B. napus*

	LOC106397267	LOC106408997	LOC106410496	LOC106414122	LOC106445255	LOC111202315
LOC106397267		87.32	87.32	86.41	98.16	86.35
LOC106408997	87.32		98.48	85.88	87.21	85.56
LOC106410496	87.32	98.48		85.99	87.10	85.67
LOC106414122	86.41	85.88	85.99		86.51	97.84
LOC106445255	98.16	87.21	87.10	86.51		86.46
LOC111202315	86.35	85.56	85.67	97.84	86.46	

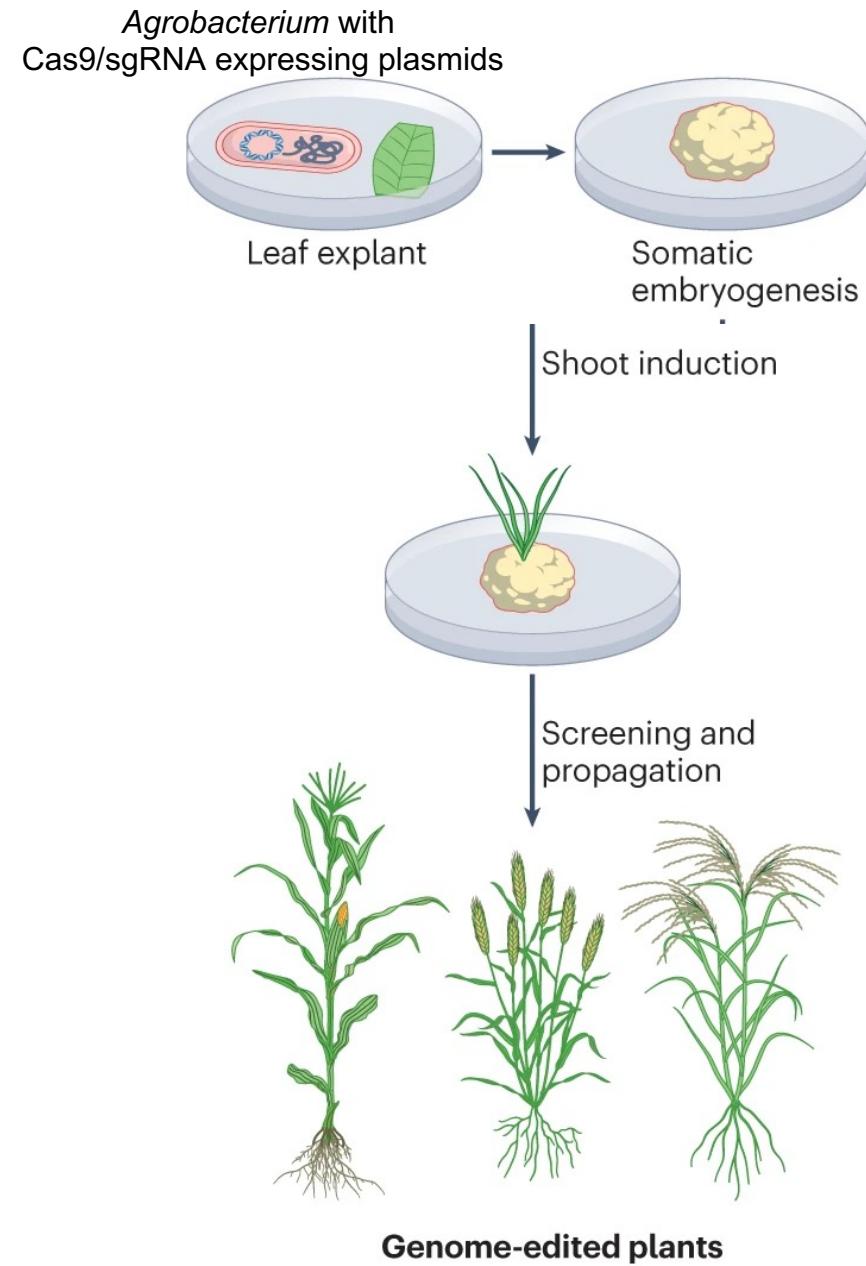
Protein identity of *GTR1* genes in *B. napus*

	LOC106397267	LOC106408997	LOC106410496	LOC106414122	LOC106445255	LOC111202315
LOC106397267		88.94	89.27	85.37	98.58	85.04
LOC106408997	88.94		99.19	86.41	89.11	85.76
LOC106410496	89.27	99.19		86.57	89.43	86.08
LOC106414122	85.37	86.41	86.57		85.53	97.56
LOC106445255	98.58	89.11	89.43	85.53		85.20
LOC111202315	85.04	85.76	86.08	97.56	85.20	

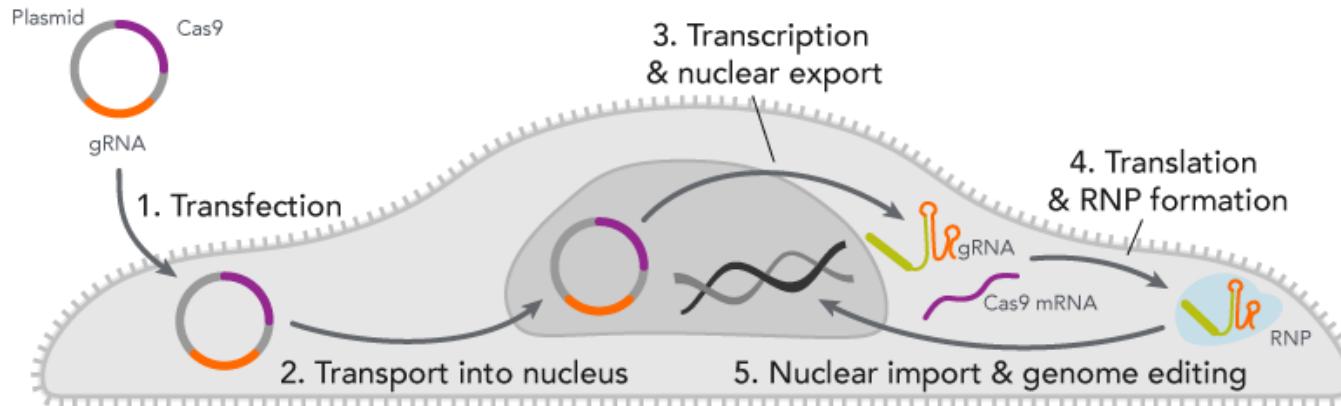
Transformation Techniques (Tissue culture dependent)



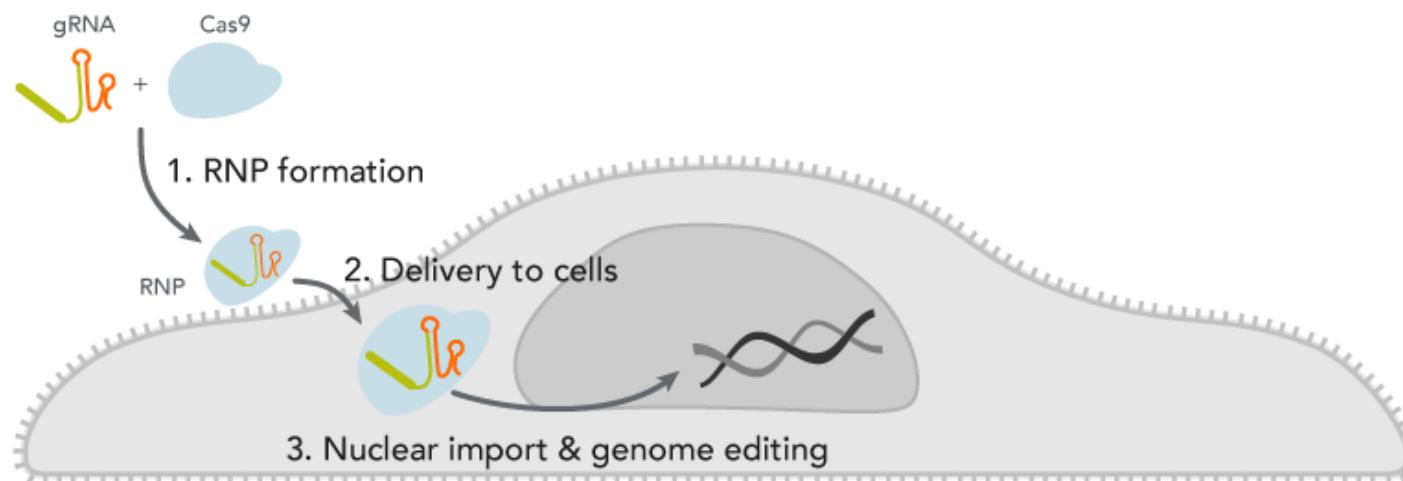
Genome editing using protoplasts
Homozygotic transgene-free mutants
at T0 generation



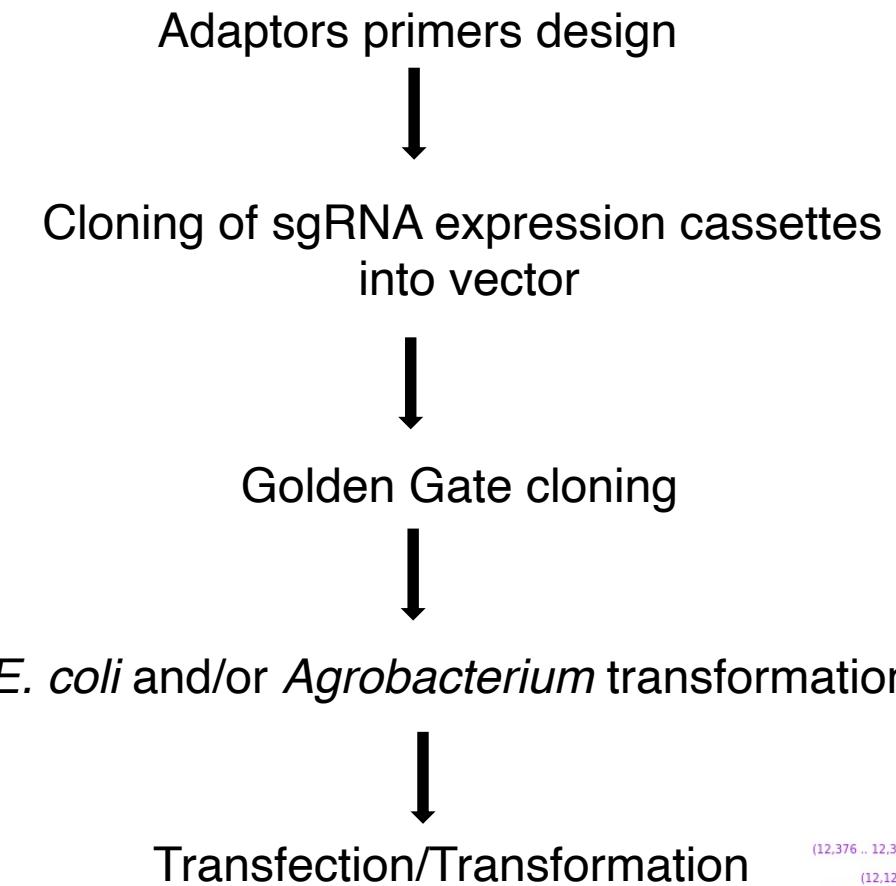
Plasmid-based editing



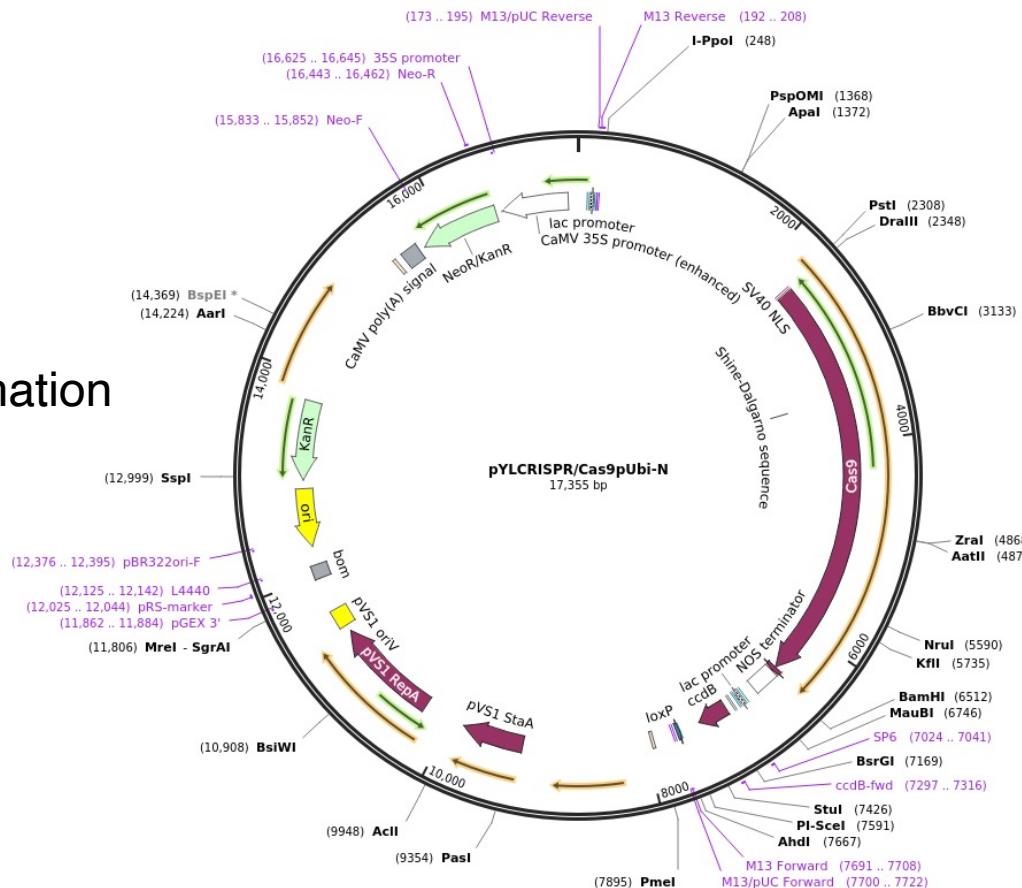
RNP-based editing



Plasmid-based editing



Vector Map



Adaptors primers design

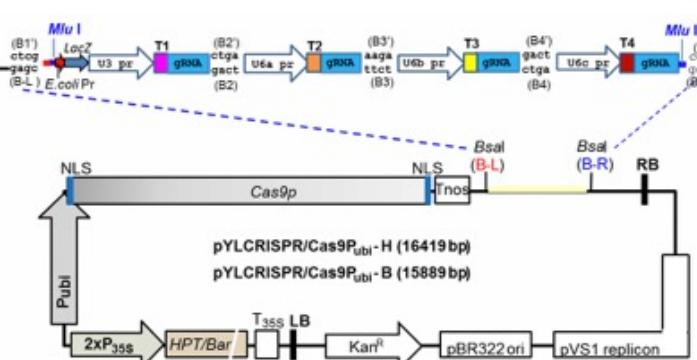
primerDesign

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CRISPR-GE targetDesign MMEJ-KO primerDesign offTarget seqDownload Help

primerDesign-V: design primers for vector construction

To prepare CRISPR/Cas9 binary constructs, the first step is to generate target-sgRNA expression cassette(s). The **primerDesign-V (Vector)** tool automatically outputs the primers for PCR-based generation of the sgRNA expression cassettes in the vector system of our lab and other vectors using the adapter-ligation method.



primerDesign

Home Contact us Site Map

CRISPR-GE targetDesign MMEJ-KO primerDesign offTarget seqDownload Help

primerDesign-V: automatically generates primers for vectors constructing of CRISPR/Cas9 genome editing in plants

Currently, the primerDesign-V tool can design primers for preparation of the sgRNA expression cassettes in the vector system of our lab and other vectors using the adapter-ligation method.

Please select the vector system for construction of sgRNA expression cassette:

The vector system of YG Liu Lab Another vector system (use the adapter ligation method).

Reference:

Ma X, Zhang Q, Zhu Q, et al., A robust CRISPR/Cas9 system for convenient high-efficiency multiplex genome editing in monocot and dicot plants. 2015, *Mol. Plant*, 8 (8), 1274-1284. [\[Link\]](#)
Ma X, Liu Y-G*. CRISPR/Cas9-based multiplex genome editing in monocot and dicot plants. *Curr. Protoc. Mol. Biol.*, 2016, 115, 31.6.1-31.6.21. [\[Link\]](#)

Please enter your target sequence (20 nt) without PAM. [\[...demo\]](#)

ID	Target sequence of CRISPR/Cas9 (5'-3')	Promoter	Method	Primers (5'-3')	Tm
1	AATGAGACATTTGAGAAGAT	AtU3d	method1	AtU3dT1F: gtcaATGAGACATTTGAGAAGAT AtU3dT1R: aaacATCTTCTCAAATGTCTCAT	58.2 56.4
2	GAATCAACAGTTCTTCAAC	AtU3b	method1	AtU3bT2F: gtcaGAATCAACAGTTCTTCAAC AtU3bT2R: aaacGTTGAAGAAACTGTTGATTC	60.4 58.7

Insert Delete Design

[Go to primerDesign-V tool](#)

<http://skl.scau.edu.cn/primerdesign/vector/>

Assembly of sgRNA expression cassettes into a CRISPR/Cas9 construct

- ❖ Preparation of sgRNA expression cassette templates
- ❖ Arrangement of sgRNA cassettes in a pYLCRISPR/Cas9 vector

1 target: LacZ-AtU3d

2 targets: LacZ-AtU3d—AtU3b

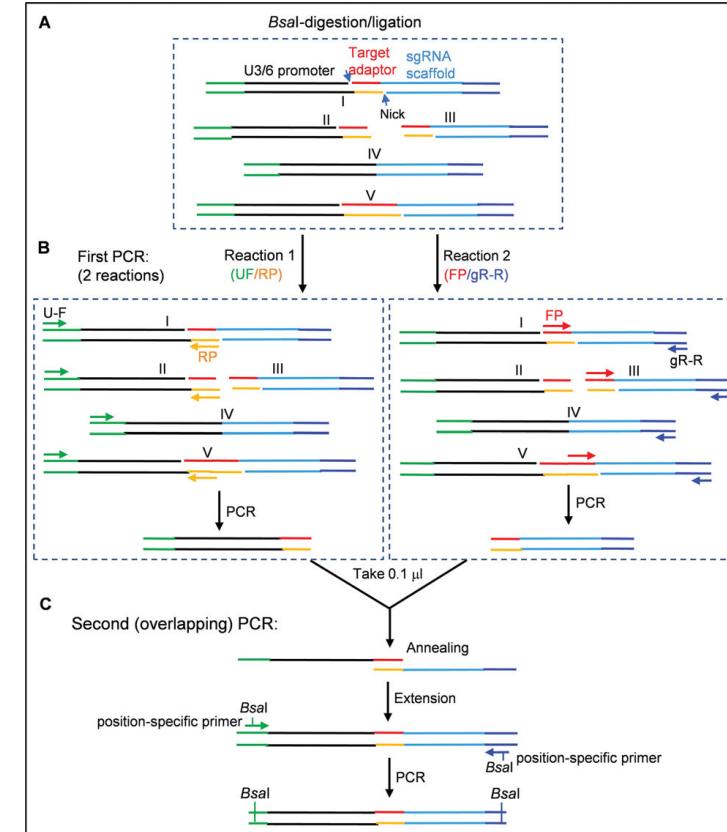
3 targets: LacZ-AtU3d—AtU3b—AtU6-1

4 targets: LacZ-AtU3d—AtU3b—AtU6-1—AtU6-29



- ❖ *E. coli* and/or *Agrobacterium* transformation

- ❖ Transfection/Transformation



A strategy for preparation of sgRNA expression cassettes

Off Target Prediction

CRISPR
RGEN Tools About Cas-OffFinder Microhomology Cas-Designer Database Analyzer Digenome-Seq Base Editing Prime Editing

Cas-OffFinder

A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases.

Citation info: Bae S., Park J., & Kim J.-S. Cas-OffFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* 30, 1473-1475 (2014).

Submit a new searching job, or [download an off-line version of Cas-OffFinder here](#).

Job title (Optional):

E-mail (Optional):

* The result will be notified by e-mail (searching job is working in sequence for many input data, therefore it would be convenient to receive the results by e-mail).

PAM Type

CRISPR/Cas-derived RNA-guided Endonucleases (RGENs)

- SpCas9 from *Streptococcus pyogenes*: 5'-NGG-3'
- SpCas9 from *Streptococcus pyogenes*: 5'-NRG-3' (R = A or G)
- StCas9 from *Streptococcus thermophilus*: 5'-NNAGAAW-3' (W = A or T)
- NmCas9 from *Neisseria meningitidis*: 5'-NNNNNGMTT-3' (M = A or C)
- SaCas9 from *Staphylococcus aureus*: 5'-NNGRRT-3' (R=A or G)
- CjCas9 from *Campylobacter jejuni*: 5'-NNNVRVAC-3' (V = G or C or A, R = A or G, Y = C or T)

Query Sequences

Query sequences (5' to 3'), one sequence per line.

Please write crRNA sequences **without PAM sequences** (e.g. without NGG for SpCas9). The length of each query sequence should be between 15 and 25 nt, and **all be the same length**:

```
CAGCAACTCCAGGGGGCGCAAGGAACCATTGTGTTAA
```

Mismatch Number (eq or less than) 0

DNA Bulge Size (eq or less than) 0

 offTarget

Home Contact us

CRISPR-GE targetDesign MMEJ-KO primerDesign offTarget seqDownload Help

offTarget: predict the potential off-target sites of Cas9/Cpf1 RNA-guided endonucleases

PAM type: SpCas9 from *Streptococcus pyogenes*: 5'-NGG-3'

* Or define your own PAM:

PAM sequence: NGG

PAM position: 3'

Guide length (nt): 20

Target (reference) genome: *Brassica napus* (Genoscope, v5)

Query

Please enter your target sequences and PAM. [\[.demo\]](#)

ID	Target sequence (5'-3')	PAM
Target sequence 1:		

Insert Delete Submit

Target Genome

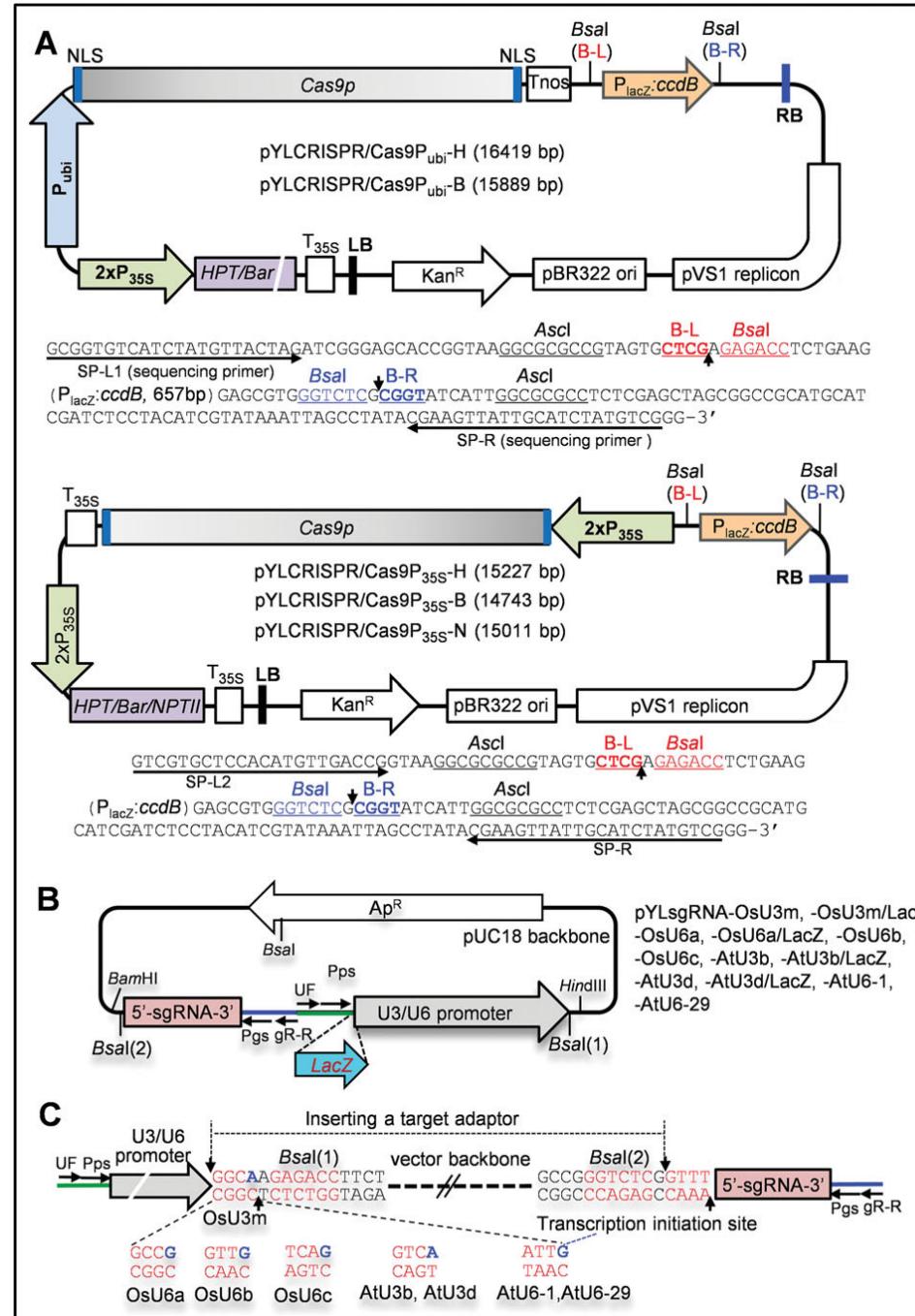
Organism Type

Plants

Genomes

- Arabidopsis thaliana* (TAIR10) - Thale cress
- Oryza sativa* (OSv4) - Rice
- Solanum lycopersicum* (SL2.4) - Tomato
- Zea mays* (AGPv3) - Corn
- Chlamydomonas reinhardtii* (Chlre4)
- Solanum tuberosum* (PGSC v4.03) - Potato
- Glycine max* (v1.0) - Soybean
- Vitis vinifera* (IGGP_12X/Ensplost26) - European grapevine
- Manihot esculenta* (JGI 4.1) - Cassava
- Malus domestica* (JGI 1.0) - Apple
- Hordeum Vulgare* (Ensembl Plants 28) - Barley
- Nicotiana benthamiana* (v1.0.1)
- Fragaria vesca* (1.0) - Wild strawberry
- Citrus sinensis* (1.0) - Sweet orange
- Theobroma cacao* (CIRAD 1.0) - Cacao
- Theobroma cacao* (CGD 1.1) - Cacao
- Solanum lycopersicum* (SL2.5) - Tomato
- Musa acuminata* (MA1) - Banana
- Arachis ipaensis* (PeanutBase v1.0)
- Arachis duranensis* (PeanutBase v1.0)
- Actinidia chinensis* (from IKG) - Kiwifruit
- Brassica napus* (v4.1) - Rapeseed
- Glycine max* (v2.0) - Soybean
- Sorghum bicolor* (v1.0) - Cereal grass

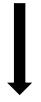
Vectors and intermediary Plasmids



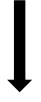
RNP-based editing

- CRISPR relies on the nuclease activity of Cas protein and their specific binding to the genome directed by guide RNAs (gRNAs)

CRISPR-endonuclease production



sgRNA production

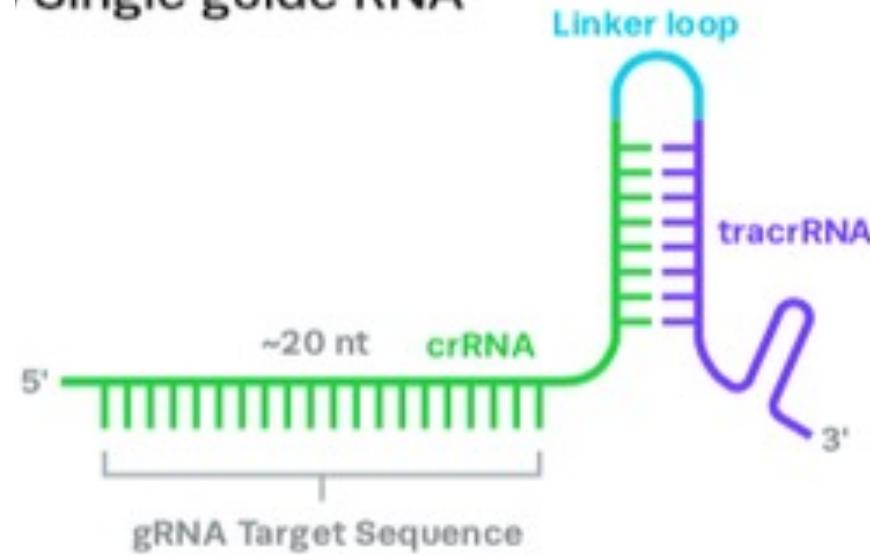


RNP assembly



Transfection to protoplasts

Single guide RNA



RNP-based editing

Why it is needed

- Transgenes can be segregated through breeding; the process is usually time- and labor-intensive
- Not a viable option for species having a lengthy juvenile growth period or vegetatively propagated plants
- Self-incompatible plants
- If genomic DNA is continuously exposed to CRISPR construct, there is the possibility of off-target mutagenesis and chimeric mutants

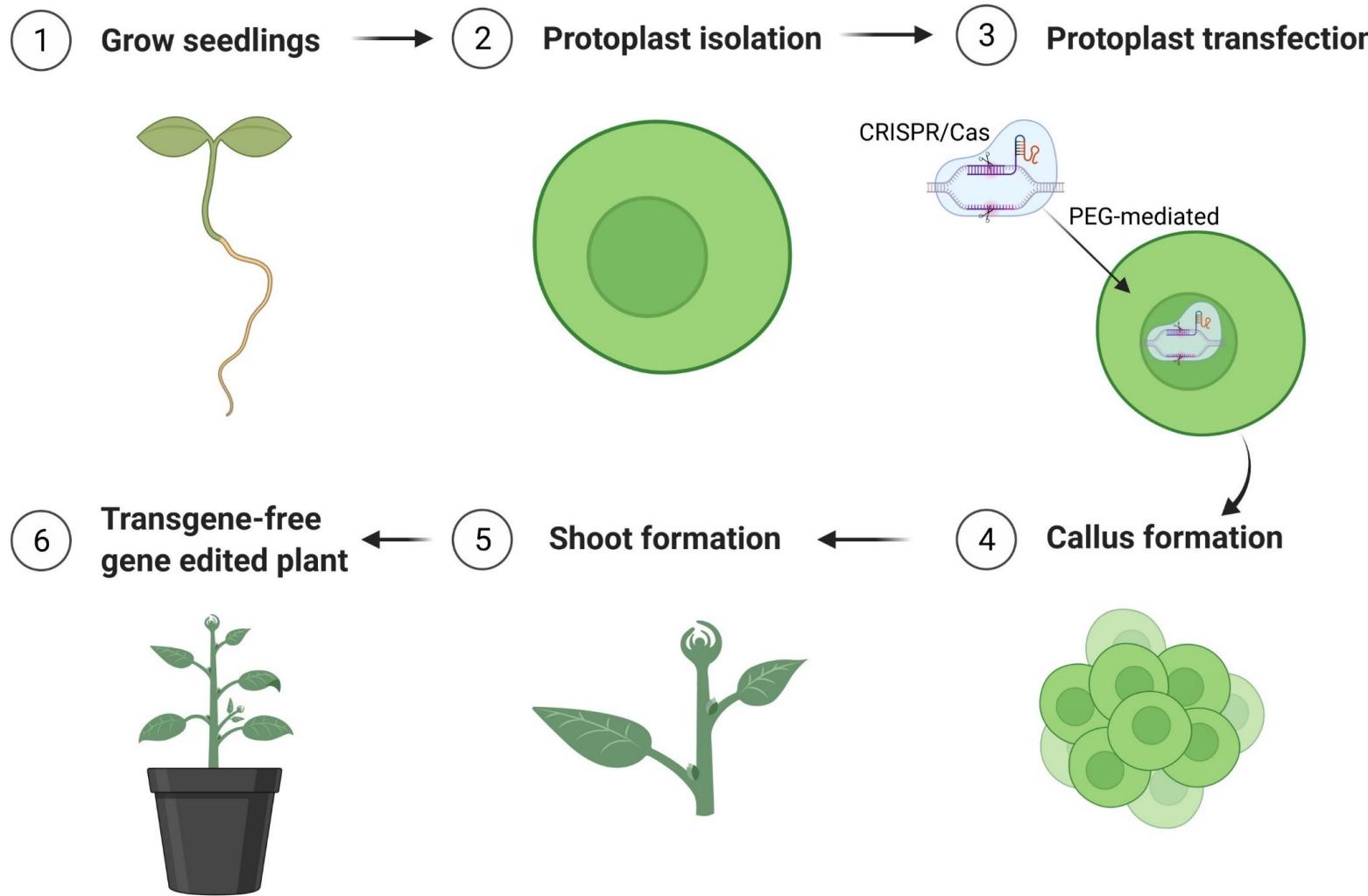
Advantages

- Enables generation of transgene-free gene-edited lines
- Minimal off-target effects
- Reduced toxicity due to the rapid degradation of RNPs
- Ability to titrate their dosage while maintaining high editing efficiency

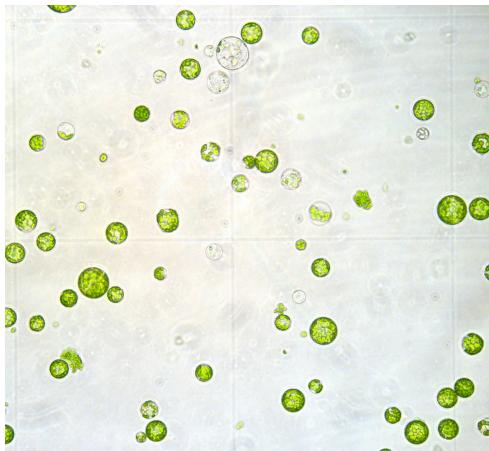
Disadvantages

- Regeneration of plants from protoplasts is a major bottleneck in most crop species
- Somaclonal Variation and Genomic Instability

Workflow for genome editing using protoplasts



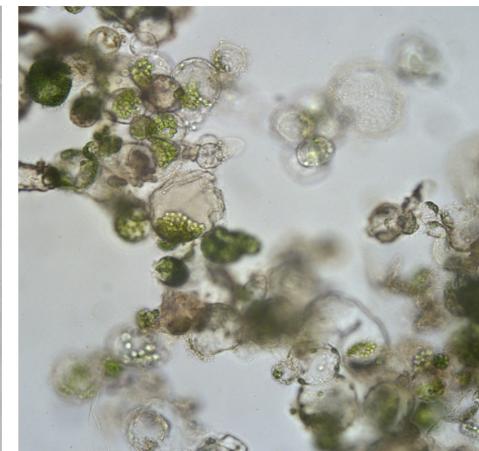
PEG-mediated protoplast transfaction/regeneration



Freshly isolated protoplasts



Protoplasts undergoing cell divisions and multiplication



Protoplast colonies



Shoot regeneration from protoplast colonies

Molecular characterization of editing events

Medium-throughput assays

- T7 Endonuclease 1 (T7E1) Assay
- HRFA

High-throughput assays

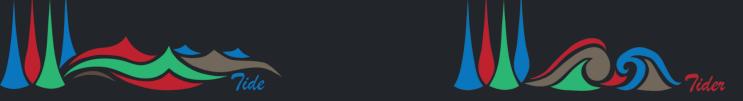
Sanger Sequencing

Tracking of Indels by Decomposition (TIDE)

ICE (inference of CRISPR edits)

NGS

RAPID AND EASY QUANTITATIVE ASSESSMENT OF GENOME EDITING



TIDE

- For non-templated Cas9 editing
- Input: 2 Sanger sequence traces
- Output: Quantitative spectrum of indels around the cut site

[READ MORE](#) | [PUBLICATION](#)

TIDER

- For template-directed Cas9 editing
- Input: 3 Sanger sequence traces
- Output: Quantification of templated mutations plus the spectrum of non-templated indels

[READ MORE](#) | [PUBLICATION](#)

[Start TIDE](#)

[Start TIDER](#)

SYNTHEGO

ICE Analysis

Get NGS quality results with Sanger data in seconds

Sample by Sample Upload Batch Upload

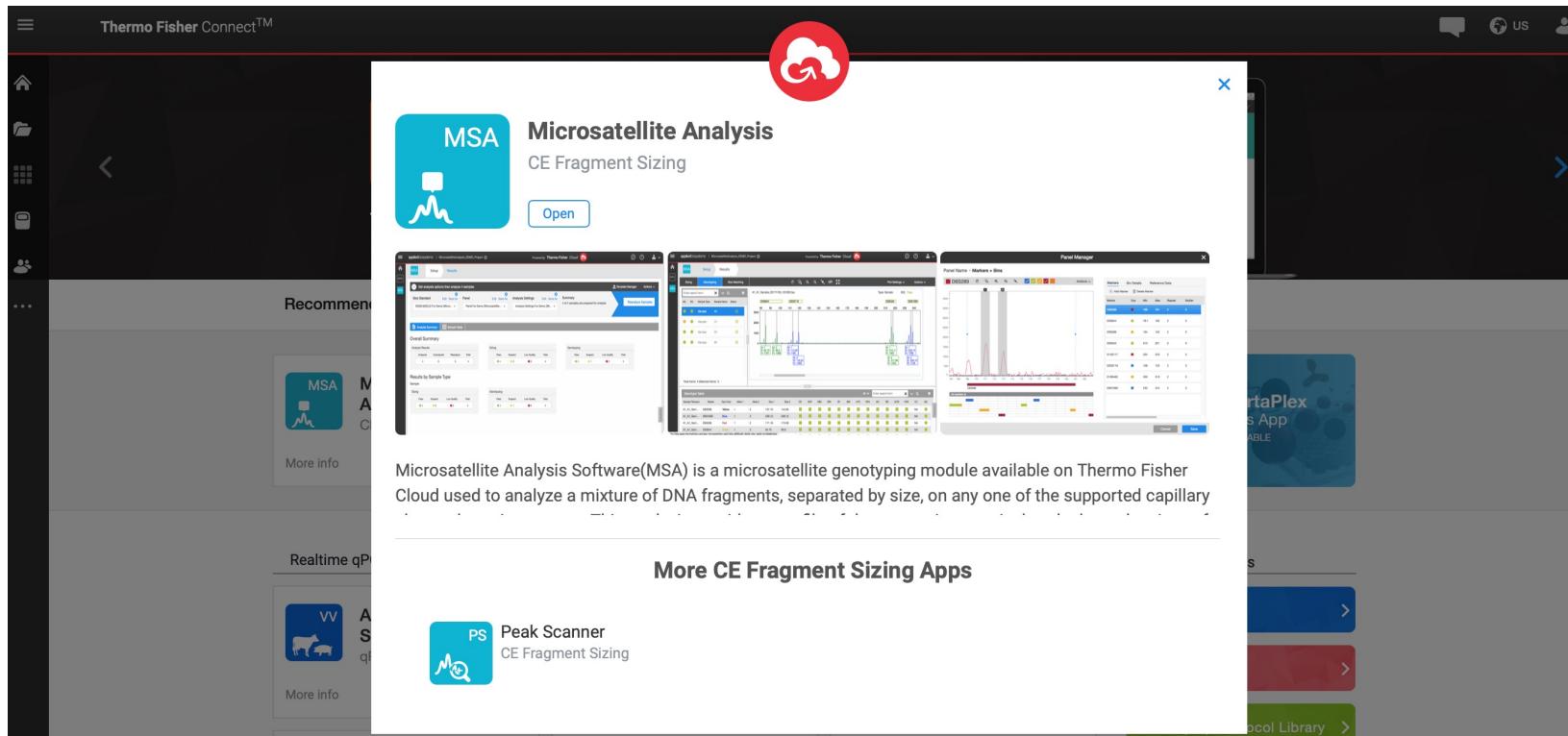
Label: Unique sample name Guide Sequences (Max 3): 17-23 nt RNA sequences without PAM Multi-guide

Donor Sequence (Optional): 16-30 nt DNA sequence with homology arms. Homology arm must have at least 15 bp of alignment. Knockin

Control File: Drop control. ab1 file here or browse your files Experiment File: Drop experiment. ab1 file here or browse your files

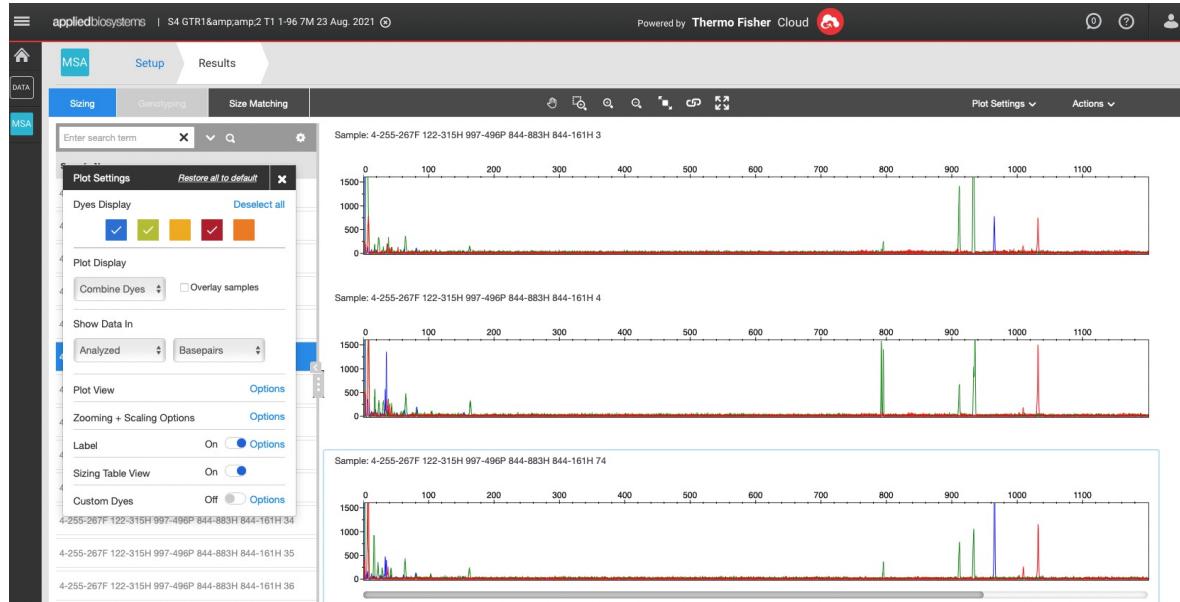
High Resolution Fragment Analysis

- ❖ 3500 Genetic analyzer
- ❖ PCR with one of the primer attached Fluorescent dyes
- ❖ 6 different dyes at the same time
- ❖ GeneMapper™ analysis
- ❖ Thermo Fisher Connect™



High Resolution Fragment Analysis

Fragment analysis using Thermo Fisher Connect™



Multiple Samples

Peak Overview

403 Peaks

20 YELLOW

204 ORANGE

145 RED

27 GREEN

7 BLUE

Edit Peaks

All Peaks

Enter search term

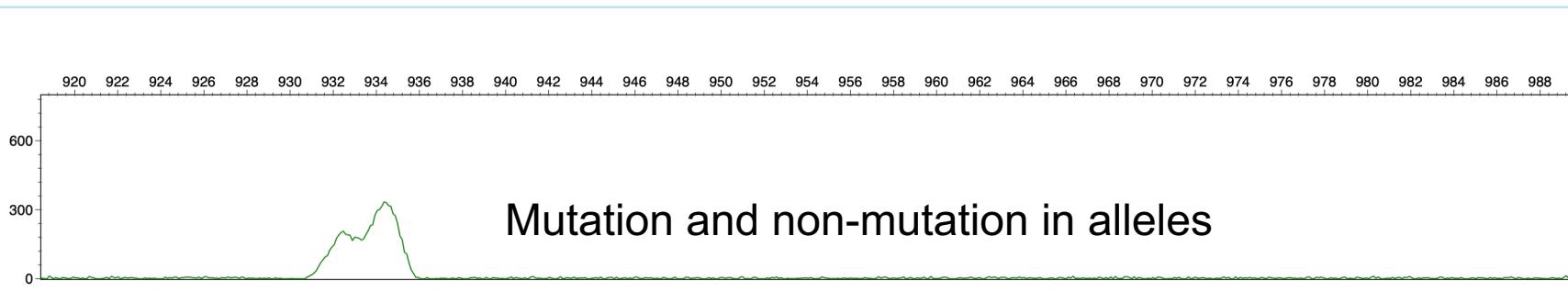
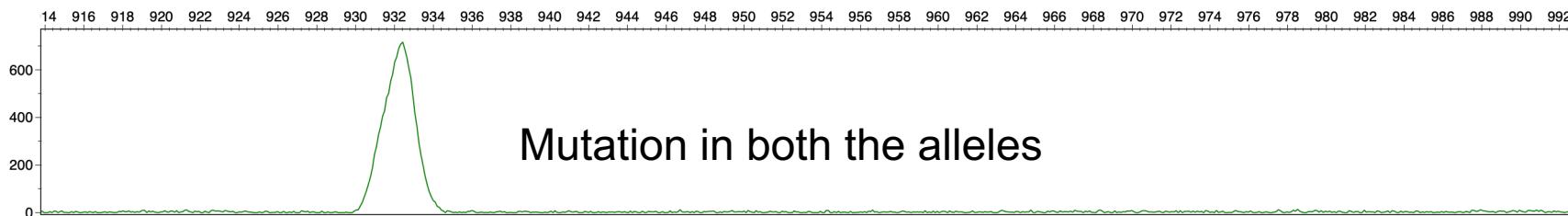
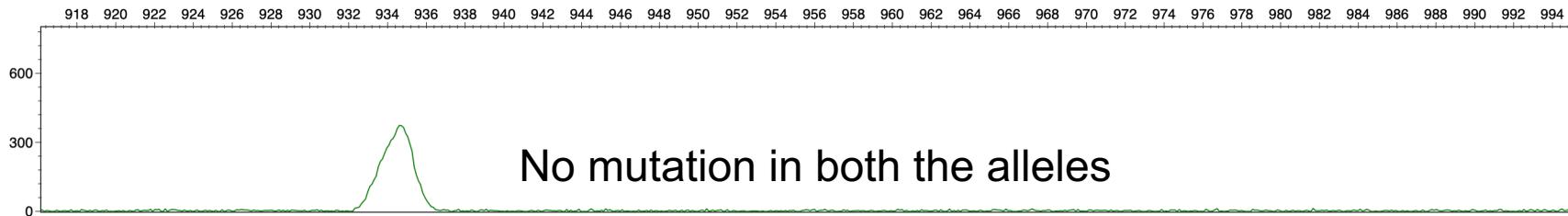
Show/Hide Columns

Dye Color	Sample Name	Size	Height	Area (Data Point)	Area (Base Pairs)	Begin Point (Base Pa...	End Point (Base Pairs)
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	993.88	74.00	350.00	38.93	993.10	995.21
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	991.88	55.00	105.00	11.67	991.43	992.21
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	987.88	83.00	313.00	34.76	986.88	988.43
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	982.00	52.00	378.00	41.91	981.22	983.10
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	980.00	145.00	2,152.00	238.18	977.90	982.00
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	980.00	200.00	2,946.00	325.22	977.79	982.87
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	980.00	169.00	2,313.00	259.92	977.41	982.36
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	979.23	69.00	217.00	24.00	978.78	979.78
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	978.23	61.00	351.00	38.73	977.46	978.78
Blue	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	964.29	507.00	6,970.00	768.10	961.54	966.38
Blue	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	963.96	155.00	2,338.00	251.24	960.96	966.66
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	963.85	51.00	424.00	45.53	962.46	964.39
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	960.00	158.00	2,311.00	243.54	957.29	962.35
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	960.00	209.00	3,181.00	340.12	957.15	963.13
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	960.00	178.00	2,438.00	265.47	957.18	961.87
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	940.00	157.00	2,099.00	216.57	938.22	942.66
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	940.00	193.00	2,722.00	284.71	937.89	942.59
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	940.00	172.00	2,199.00	232.07	938.31	942.53
Yellow	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	934.41	175.00	2,641.00	277.89	933.25	936.30
Green	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	934.30	334.00	5,566.00	585.51	933.04	935.99

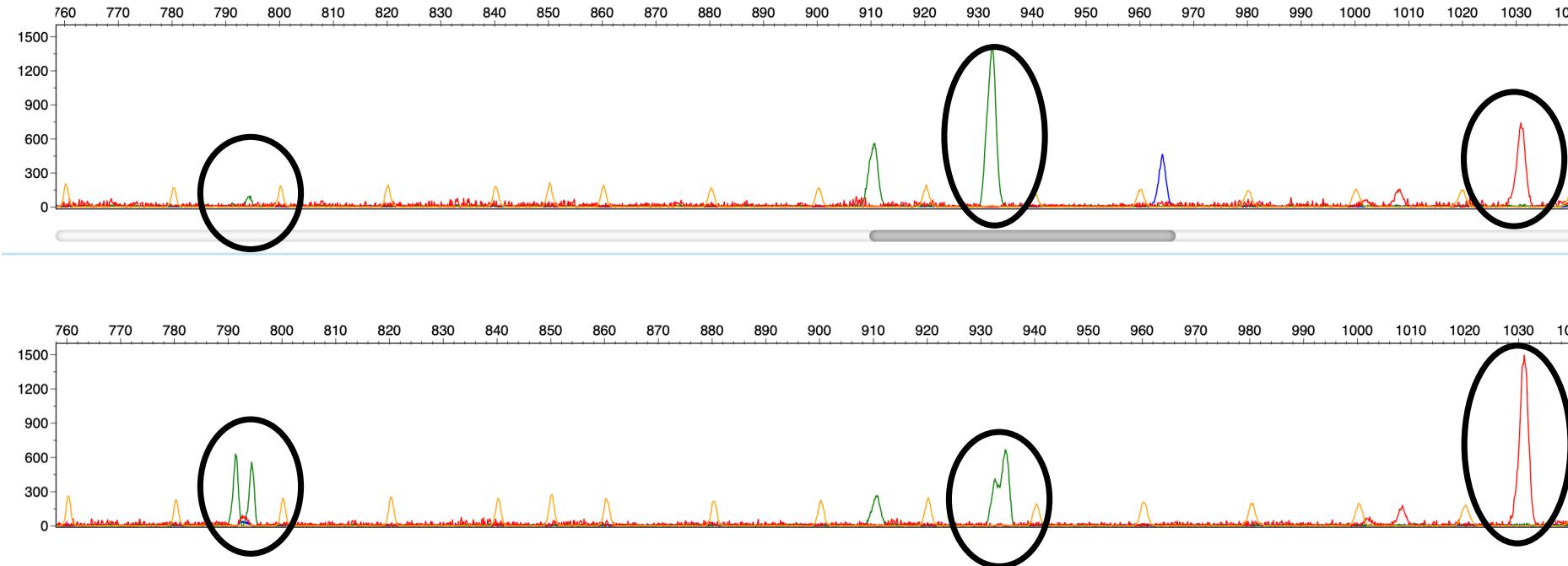
Restore to default

Apply

High Resolution Fragment Analysis



High Resolution Fragment Analysis

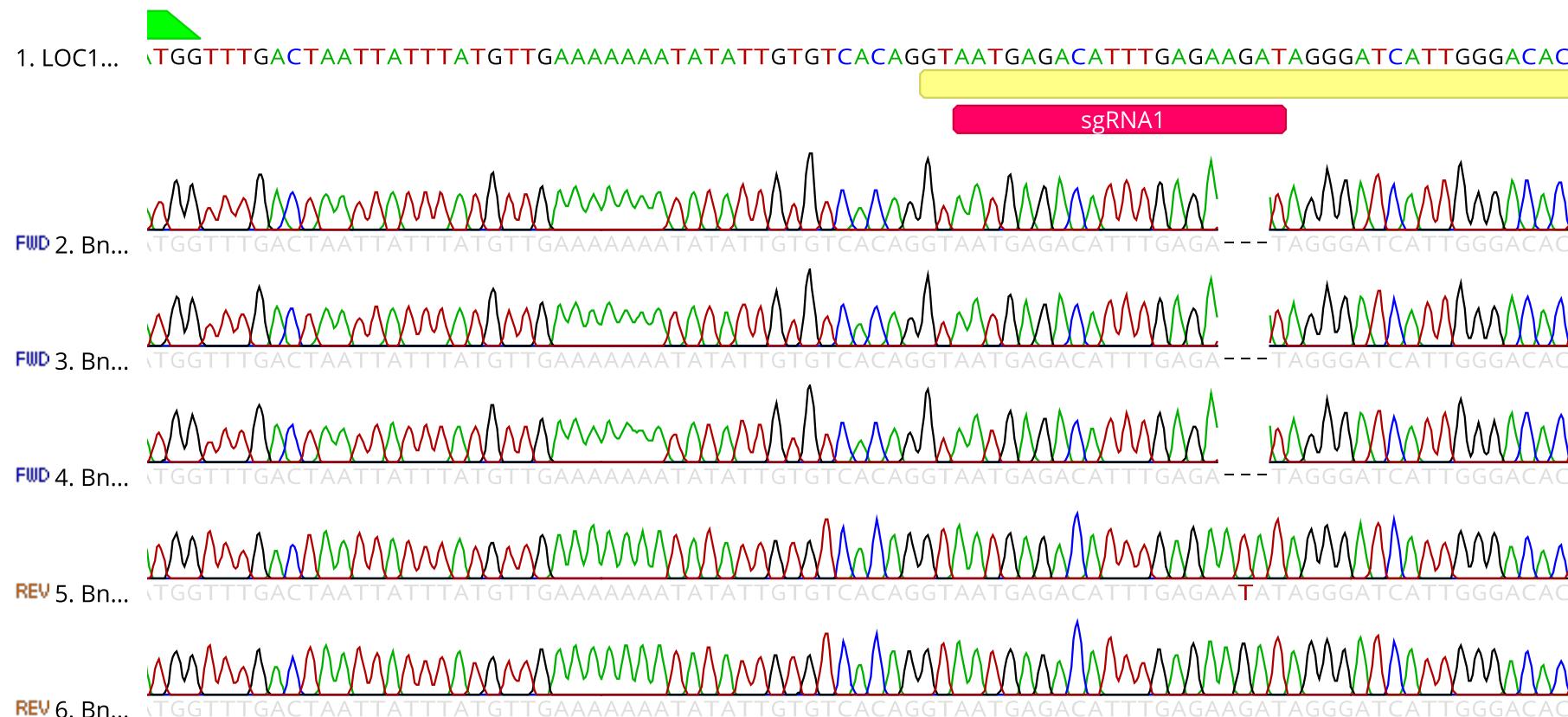


Multiple PCR products in one sample

Sanger Sequencing Result Analysis

PCR with gene-specific primers amplifying gRNA targeting DNA

Cloning and transformation



Molecular characterization of editing events

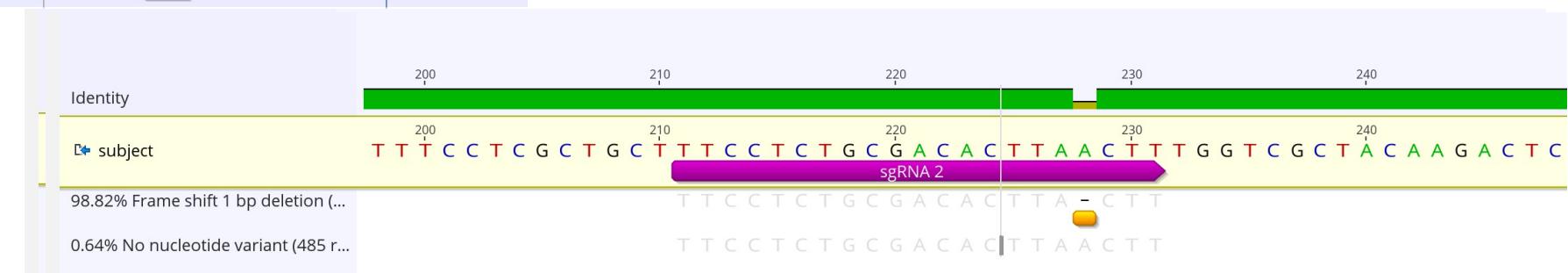
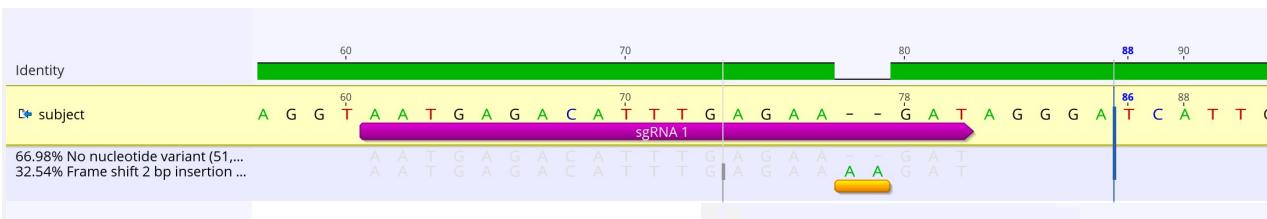


CRISPResso2

Analysis of genome editing outcomes from deep sequencing data

CRISPResso2

geneious prime



NGS-Illumina-Next Generation Sequencing Technologies

Amplicon sequencing Analysis

Cas-Analyzer

A JavaScript-based instant assessment tool for high-throughput sequencing data for genome edited cells

Thanks to the improvements in the newer JavaScript engines in the most recent web browsers, the Cas-Analyzer basic internal algorithm of Cas-Analyzer completely runs on the client-side so that large amounts of sequencing data do not need to be uploaded to the server. Currently, Cas-Analyzer supports various single nucleases (SpCas9, StCas9, NmCas9, SaCas9, and AsCPf1) and paired nucleases (ZFNs, TALENs, aCas9 nickases, and dCas9-fok nucleases).

Citation info: Park J. *et al.* Cas-Analyzer: an online tool for assessing genome editing results using NGS data. *Bioinformatics* **33**, 286-288 (2017).

For the ones who would like to clarify the errors derived from DNA polymerase during PCR or sequencing process, comparison of treated sample and negative control (e.g. untreated sample) is recommended. Please input your data in below form, or [download an example data here](#).

Sequencing Data

File Type: Paired-end reads

Read 1 (fastq or gzipped fastq):

Read 2 (fastq or gzipped fastq):

Basic Information

Full reference sequence (5' to 3'):

Nuclease Type:

Select Nuclease:

Analysis Parameters

Comparison range (R) or use both ends

Minimum frequency (n)

(Optional) WT marker (r)

Regulatory Landscape of Genome Editing

- ❖ Aim of plant breeding is to find or generate new genetic variation by searching for different alleles in germplasms or induced mutagenesis using chemicals or physical mutagens
- ❖ Regulation in North America, South America, and Asia-Pacific region rapidly approving gene-edited crops, especially crops without external DNA having no off-target edits
- ❖ On July 5, 2023, the EU adopted a proposal to deregulate the approval of gene-edited crops under New Genomic Technologies (NGT)
- ❖ NGTs are currently under debate in EU parliament with some specifications, such as
 - Up to 20 different independent genetic changes
 - Substitution or insertion of no more than 20 nucleotides
 - Deletion of any number of nucleotides
 - Modification does not interrupt an endogenous gene, etc.
 - No risk assessments, mandatory traceability, or labelling on the end product as compared to transgenic crops developed in earlier biotechnology techniques
 - Gene-editing crops can not patentable

Conclusion

- ❖ Limited genetic diversity in available elite cultivars/germplasms
- ❖ CRISPR/Cas9 could be a game-changer in modern plant breeding
- ❖ Targeted gene modifications can be obtained more precisely and faster than conventional plant breeding techniques
- ❖ Need to find out the novel traits and genes for biofortification to address the hidden hunger
- ❖ While challenges in genome editing in many crops, and advancements in nutritional biotechnology are paving the way

Questions?

selvaraju.kanagarajan@slu.se