

Transgene (DNA)-Free Genome Editing



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Department of Plant Breeding

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

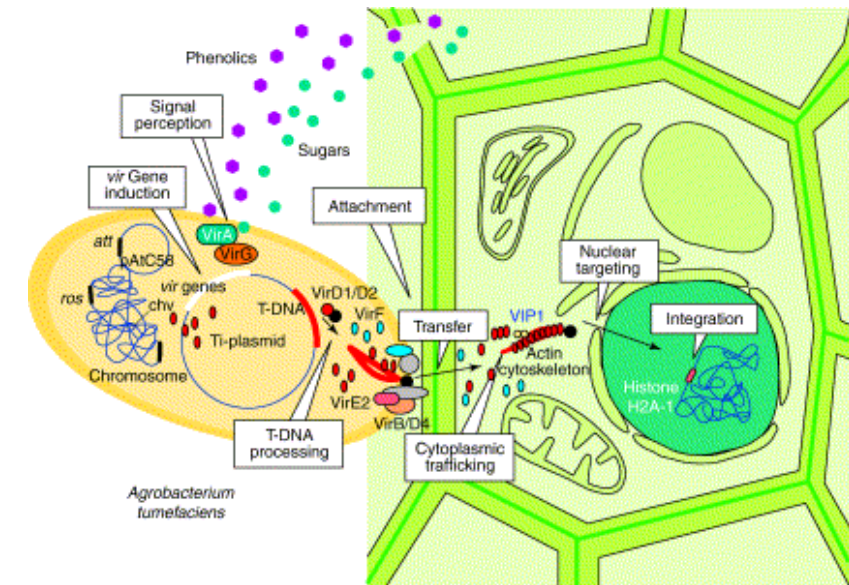
- ❖ Application of traditional breeding methods combined with emerging molecular biology techniques
- ❖ Development of Tissue Culture Techniques

Early Genetic Engineering

- Genetic Transformation
- Development of Transgenic Plants



www.plantcelltechnology.com



Gelvin, 2003

Plant cell

TRENDS in Biotechnology

Second Generation of Biotechnology

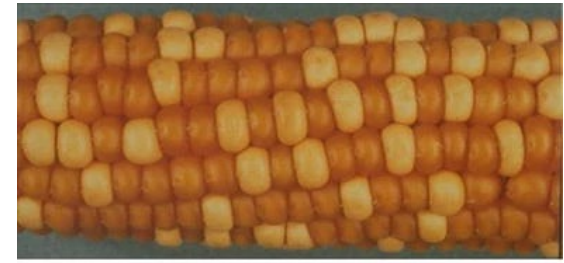
Represents more sophisticated techniques and applications, particularly in genetic engineering and molecular biology.

Key Aspects

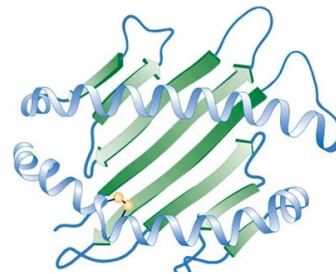
Advanced Genetic Engineering- Creation of genetically modified organisms (GMOs) with specific traits and more complex manipulations of DNA, like gene silencing & gene editing

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

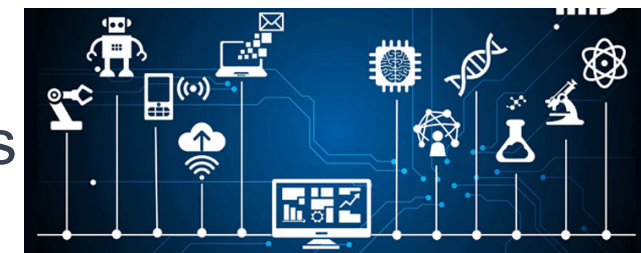
Bioinformatics and Computational Biology- Using computational tools to manage and analyze biological genomics and metabolomics data and understanding biology



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

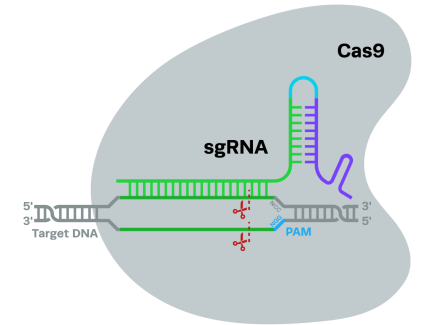
Advanced Gene Editing Technologies

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

Biofortification

Metabolic Engineering

Use of NanoPhytoBiotechnology



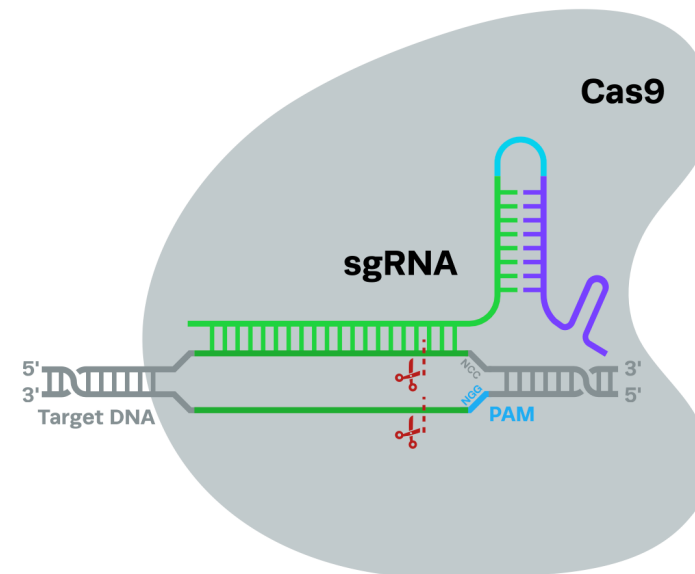
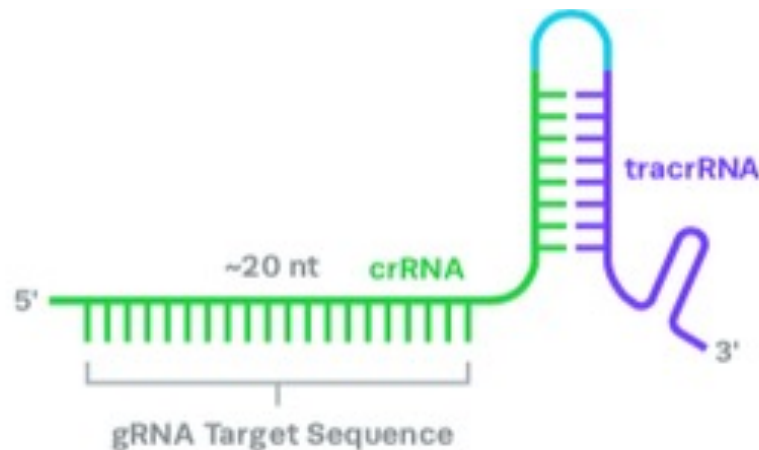
<https://www.svnthedo.com/>



Jiang et al. 2021

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 $\mu\text{mol/g}$ 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%

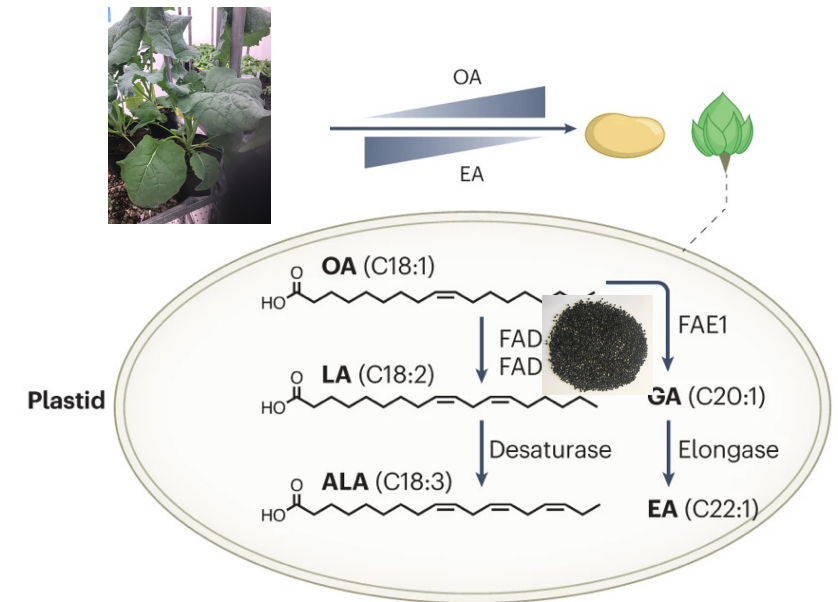
Protein ~ 20%

Fiber ~14%

Carbohydrates < 10-15%

Vitamins E & K

Minerals



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

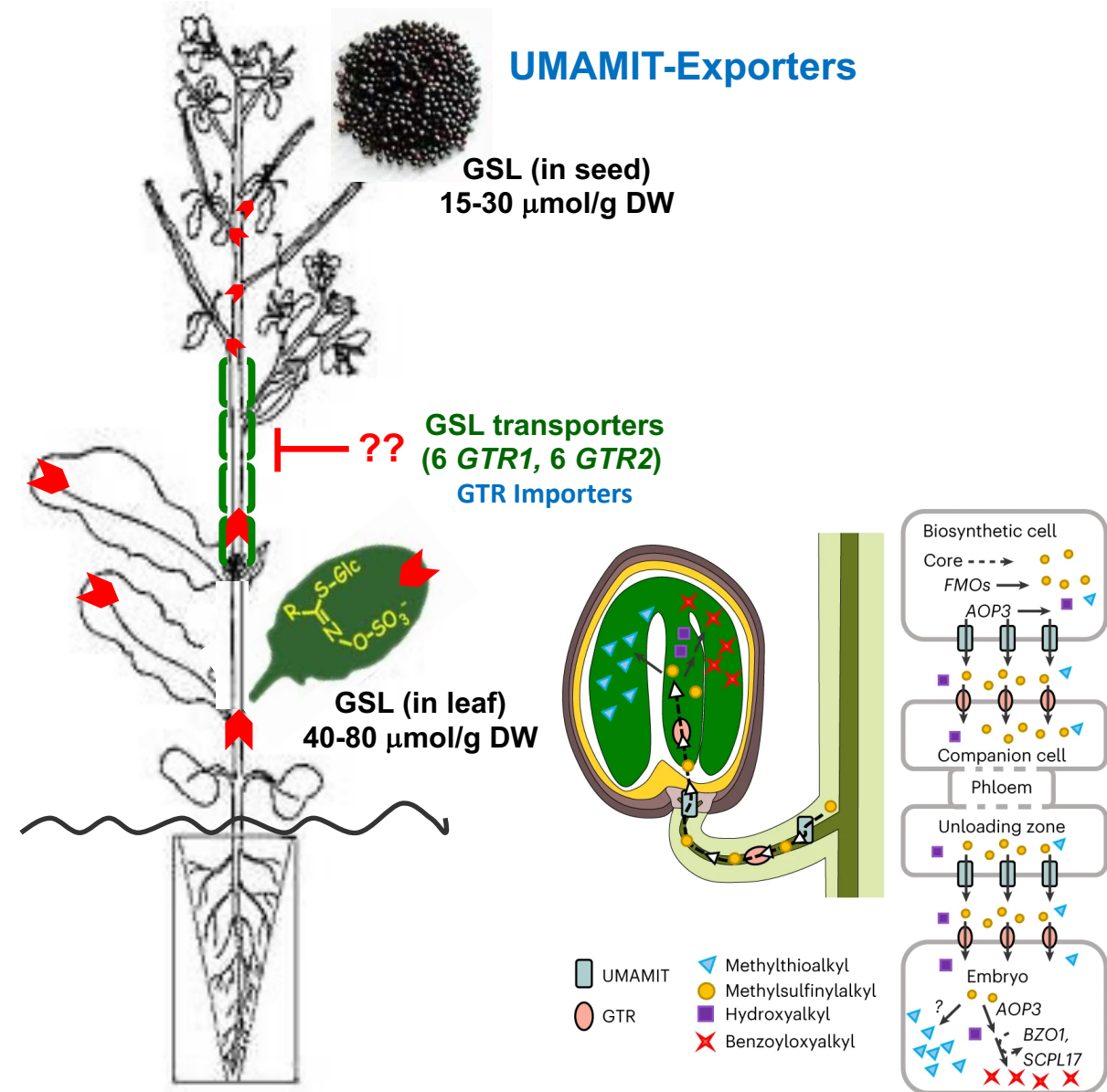
Anti-Nutritional Factors

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



Long-distance glucosinolate transport

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



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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:

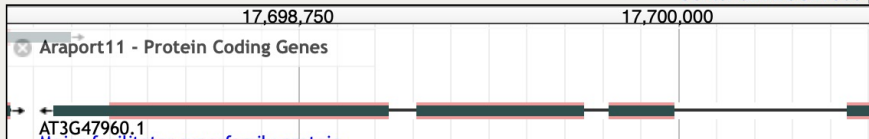
ATNPF2.10, GLUCOSINOLATE TRANSPORTER-1, GTR1, NPF2.10, NRT1/ PTR FAMILY 2.10

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.

Map Detail Image

Center on AT3G47960 | Full-screen view



Annotations

category relationship type keyword

Arabidopsis database

GTR1 gene

agtgtaaaaatgggacctataataatccagctactactcagccacacattaaaaagaacgcttaaaagggaagataaatagaataggctcagctcagagac

17700900 agatgtctctatctctccagcaaaatccacacctctttttatctttccctctcccgagctcagataaaatccagctgtgtgttattgttaccacac

17700700 ttacacacacgggttgatactatagaggaagaagcctctctggagttggaccactctactacagcagctaacacacggatgggtgattctctctggaggaaggagc

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CCGATGGAAAGCGTTCGATCTGAGTGAACATATGACGATTCGAGCAGTGGAGAAGATGAATGCAATTGAAGAGTGATTCTCAATCTGTTGTTGATCTCAC

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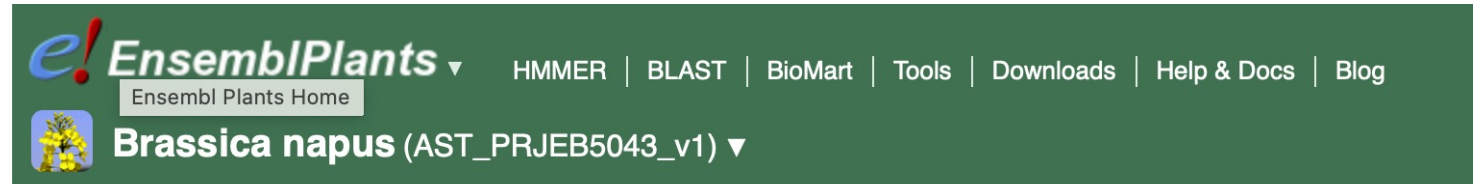
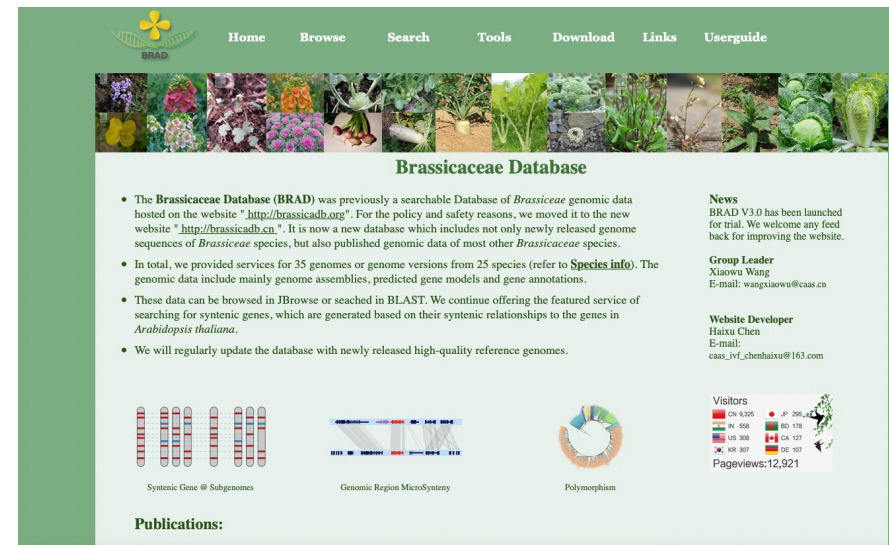
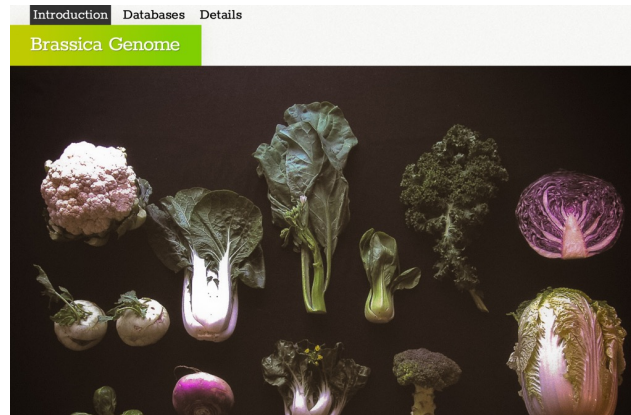
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tattgtaaaaaagttttttgtgtctaattcaaocttcgtgaagttttctgtttctctcttctatccaccacacagacgaacatacaaacacgcttaa

Brassica napus databases



Brassica napus Assembly and Gene Annotation



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

Genomes	
Assembly / Genome	NCBI Datasets 16
BioCollections	0

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

Clinical	
ClinicalTrials.gov	0
ClinVar	0

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

PubChem	
BioAssays	119
Compounds	2

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National Library of Medicine

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Genome

BETA

Download a genome data package including genome, transcript and protein sequence, annotation and a data report

Selected taxa

Brassica napus (rape) Enter one or more taxonomic names

Filters

DownloadSelect columns4 genomesRows per page201-4 of 4

<input type="checkbox"/>	Assembly	Scientific name	Modifier	Annotation	Size (Mb)	Level	Year	Action
<input type="checkbox"/>	Da-Ae (reference) RefSeq: GCF_020379485.1 GenBank: GCA_020379485.1	Brassica napus rape	Da-Ae cultivar	NCBI RefSeq Submitter	1,001	Chromosome	2021	⋮
<input type="checkbox"/>	Bra_napus_v2.0 RefSeq: GCF_000686985.2 GenBank: GCA_000686985.2	Brassica napus rape	ZS11 cultivar	NCBI RefSeq	975.8	Chromosome	2017	⋮
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<input type="checkbox"/>	ASM1417057v1 GenBank: GCA_014170575.1	Brassica napus rape	CLR6430 isolate		768.2	Scaffold	2020	⋮

blastnblasttblastntblastx

BLASTN programs search nucleotide databases using a nucleotide query. more

Enter Query Sequence

Enter accession number(s), g(s), or FASTA sequence(s) Clear

Query subrange

From

To

Or, upload file

Choose File

no file selected

Job Title

Enter a descriptive title for your BLAST search

Align two or more sequences

Choose Search Set

Database

Standard databases (nr etc.): rRNA/ITS databasesGenomic + transcript databasesBetacoronavirus

Nucleotide collection (nr/nt)

Organism

Brassica napus (taxid:3708)

exclude

Exclude

Models (XM/XP)Uncultured/environmental sample sequences

Limit to

Sequences from type material

Entrez Query

Enter an Entrez query to limit search

Program Selection

Optimize for

Highly similar sequences (megablast)More dissimilar sequences (discontiguous megablast)Somewhat similar sequences (blastn)

NCBI database for *B. napus*

Descriptions

Graphic Summary

Alignments

Taxonomy

Sequences producing significant alignments

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New Select columns

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10

?

☒ select all
 10 sequences selected

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[Graphics](#)
[Distance tree of results](#)

New

[MSA Viewer](#)

	Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover
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<input checked="" type="checkbox"/>	PREDICTED: Brassica napus protein NRT1/ PTR FAMILY 2.10-like (LOC106410496), misc_RNA	Brassica napus	rape	3708	1912	1912	90%
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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	Expect	Identities	Gaps	Strand
1912 bits(1035)	0.0	1509/1743(87%)	12/1743(0%)	Plus/Plus
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Sbjct 430		CTTTATCATTGGTAATGAGACATTTGAGAAGATAGGGATCATTTGGGACACTATCAAACCT	489	
Query 267		TCTTGGTACCTAACTTCTGTATTCAACCTTAAGAGCTACACAGCTGCAACTATCATCAA	326	
Sbjct 490		TCTGGGTACCTAACTTCAGTATTCAACCTCAAGAGTGTTACAGCTGCAACCATCATCAA	549	
Query 327		TGCCTTTAGTGGTACAATCAATTTCTGGGACTTTTATTGCTGCCTTTCTTTGCGACACTTA	386	
Sbjct 550		CGCCTTCAGTGGCACTATCAACTTCGGCACTTTCCCTCGCTGCTTTCCTCTGCGACACTTA	609	
Query 387		CTTTGGTCGCTACAAGACTCTCAGTGTCTGCTGTCATCGCTTGTTTTCTGGGATCGTTTGT	446	
Sbjct 610		CTTTGGTCGCTACAAGACTCTCTCTGTTGCTGTCTCGCTGTTTCTGGGATCGCTTGT	669	
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Sbjct 670		GATACTACTGACGGCTGCAGTTCAGGATTGCACCCCATTCCTTGTTGGAACACAAAGT-	728	

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







NCBI Reference Sequence: XM_013837850.2										
BLAST	Graphics									
to: ☺										
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ORCE	RefSeq.									
ORGANISM	Brassica napus (rape)									
	Brassica napus									
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MENT	MODEL REFSEQ: This record is predicted by automated computational analysis. This record is derived from a genomic sequence (NC_027762.2) annotated using gene prediction method: Gnomon.									
	Also see: Documentation of NCBI's Annotation Process									
On Oct 4, 2017 this sequence version replaced XM_013837850.1 .										
## Genome-Annotation-Data-START##										
Annotation Provider		: NCBI								
Annotation Status		: Full annotation								
Annotation Version		: Brassica napus Annotation Release 101								
Annotation Pipeline		: NCBI eukaryotic genome annotation pipeline								
Annotation Software Version		: 7.4								
Annotation Method		: Best-placed RefSeq; Gnomon								
Features Annotated		: Gene; mRNA; CDS; ncRNA								
## Genome-Annotation-Data-END##										
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18










B. napus pan-genomic database



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 Genes Knowledge Of Genes In Rape	 Species 1689 Rapeseed Accessions Information	 Gene Expression ~100 Thousand Genes Expression Level
 Transposable Elements Rape Repeat Database	 Population Variation ~50 Million Variations	 NLR Genes ~Three Thousand NLRs

 Gbrowse Synteny Genome Synteny And Gene Index	 ZS11 Genome Browser For ZS11	 Gangan Genome Browser For Gangan
 Westar Genome Browser For Westar	 Shengli Genome Browser For Shengli	 Tapidor Genome Browser For Tapidor
 Zheyu7 Genome Browser For Zheyu7	 No2127 Genome Browser For No2127	 Quinta Genome Browser For Quinta

 Gene Index Find Colinear Orthologous Genes	 Blast Find Similar Genes Or Sequence	 KEGG/GO Enrichment Enrichment
 Homologous Regions Interactive Homologous Region Viewer	 Orthologous Paralogs And Orthologous In Brassica	 Phylogenetic Tree Phylogenetic Analysis Of 1688 Accessions
 Seq_fetch Fetch Sequence By Location And Name	 Literature 10 Thousand Related Paper	 Genome Facts Source And Facts

Accessions	ZS11	Westar	No2127	Zheyu7	Gangan	Shengli	Tapidor	Quinta
Gene Number	100,919	97,514	95,385	96,209	96,843	94,586	96,117	95,492

BLAST program: BLASTN: NT query, NT db

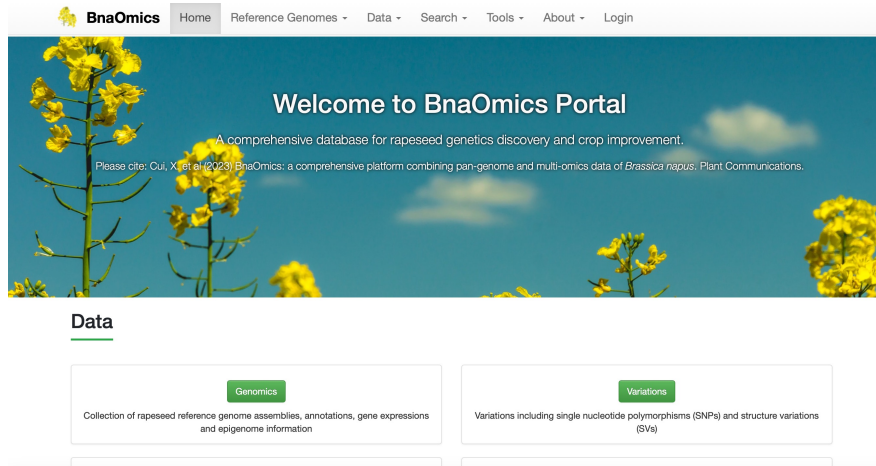
Organism: ☒ zs11 ☐ westar ☐ tapidor ☐ no2127 ☐ darmor ☐ Bra ☐ Bol

Database: zs11_CDS-(UTR-intron)(NT)

Query sequence: ATGAAGAGCAGAGTCATCCACAGCCATAGAGAGAGAAAGATAAGCATAATTACACACAGATTGAAACAATGGAGAGAAAGGCCCTTTGATGTTGAGACAACCTGAGGATCACAAAACCCCTACTCCTCCGTCGATGGCGGGTGGTTCCTGATTCGGTTCGATTTCATTTGAAGAGCAGCAGCAAAAAAGCTCGTTTATAGAGGCTGGAAGAGTCATGCCCTTTATCATTTGGTAATGAGACATTTGAGAAGATAGGGATCATTTGGGACACTATCAAACCTTCTGGTGTTACCTAACTTCAGTATTCACCTCAAGAGTGTTACAGCTGCAACCATCATCAACGCCTTCAGTGGCACTATCAACTTCGGGCACTTTCCTCGCTGCTTTCC

Description	Max score	Total score	Query cover	E value	Ident
BnaA86T0259080NO	3519	3519	100%	0	100%
BnaC03T0484480NO	3342	3342	100%	0	100%
BnaA86T0166580NO	2243	2243	93%	0	89%
BnaC03T0534680NO	2237	2237	96%	0	89%
BnaA17T0152080NO	2097	2097	95%	0	88%
BnaC05T0292180NO	2075	2075	95%	0	87%
BnaC03T046280NO	1129	1129	88%	0	79%
BnaA86T0281780NO	1118	1118	88%	0	79%
BnaC02T0468380NO	1111	1111	89%	0	79%
BnaA27T0392380NO	1091	1091	94%	0	78%
BnaC09T0842380NO	1067	1067	88%	0	78%

	Score 3519 bits(1985)	Expect 0	Identities 1905/1905(100%)	Gaps 0/1905(0%)	Strand Plus/Plus
Query 1	ATGAAGAGCAGGATCATCCACGCCATAGAGAGAGAGATGAACATTAATCACACAG	60			
Sbjct 1	ATGAAGAGCAGGATCATCCACGCCATAGAGAGAGAGATGAACATTAATCACACAG	60			
Query 61	ATTGAACAATGGAGAGAAAGCCCTTTGATGTGGAACAACATGAGATCACAAACTCTAC	120			
Sbjct 61	ATTGAACAATGGAGAGAAAGCCCTTTGATGTGGAACAACATGAGATCACAAACTCTAC	120			
Query 121	CCCTCCGTGATGGCGGTGGTTGATTCGGTCGATTCAATTTGAAGCAGCAGAAAAAG	180			
Sbjct 121	CCCTCCGTGATGGCGGTGGTTGATTCGGTCGATTCAATTTGAAGCAGCAGAAAAAG	180			
Query 181	CTCGTTTATAGAGGCTGAAAGCTACGCCCTTTATCATTTGGTAATGAGACATTTGAGAG	240			
Sbjct 181	CTCGTTTATAGAGGCTGAAAGCTACGCCCTTTATCATTTGGTAATGAGACATTTGAGAG	240			



Database Name	Source
Brassica napus pan-genome v3	
Brassica napus Westar genome	
Brassica napus Kale genome	
Brassica napus Mendel genome	
Brassica napus LT genome	
Brassica napus ZS11 genome FAFU	Chen et al. 2020
Brassica napus ZS11 genome HZAU	Song et al. 2020
Brassica napus ZS11 genome v201608	Sun et al. 2017
Brassica napus Darmor genome v4.1	Chalhoub et al. 2014
Brassica napus Darmor genome v10	Rousseau-Gueutin et al. 2020
Brassica napus Express617 genome v1	Lee et al. 2020
Brassica napus NY7 genome v2	Zou et al. 2019
Brassica napus ganganF73 genome	Song et al. 2020
Brassica napus no2127 genome	Song et al. 2020
Brassica napus quintaA genome	Song et al. 2020
Brassica napus shengli3 genome	Song et al. 2020
Brassica napus tapidor3 genome	Song et al. 2020
Brassica napus westar genome HZAU	Song et al. 2020
Brassica napus zheyu73 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



DNA extraction and PCR



Agarose gel electrophoresis



Gel extraction of PCR product



Cloning into vector and *E. coli* transformation

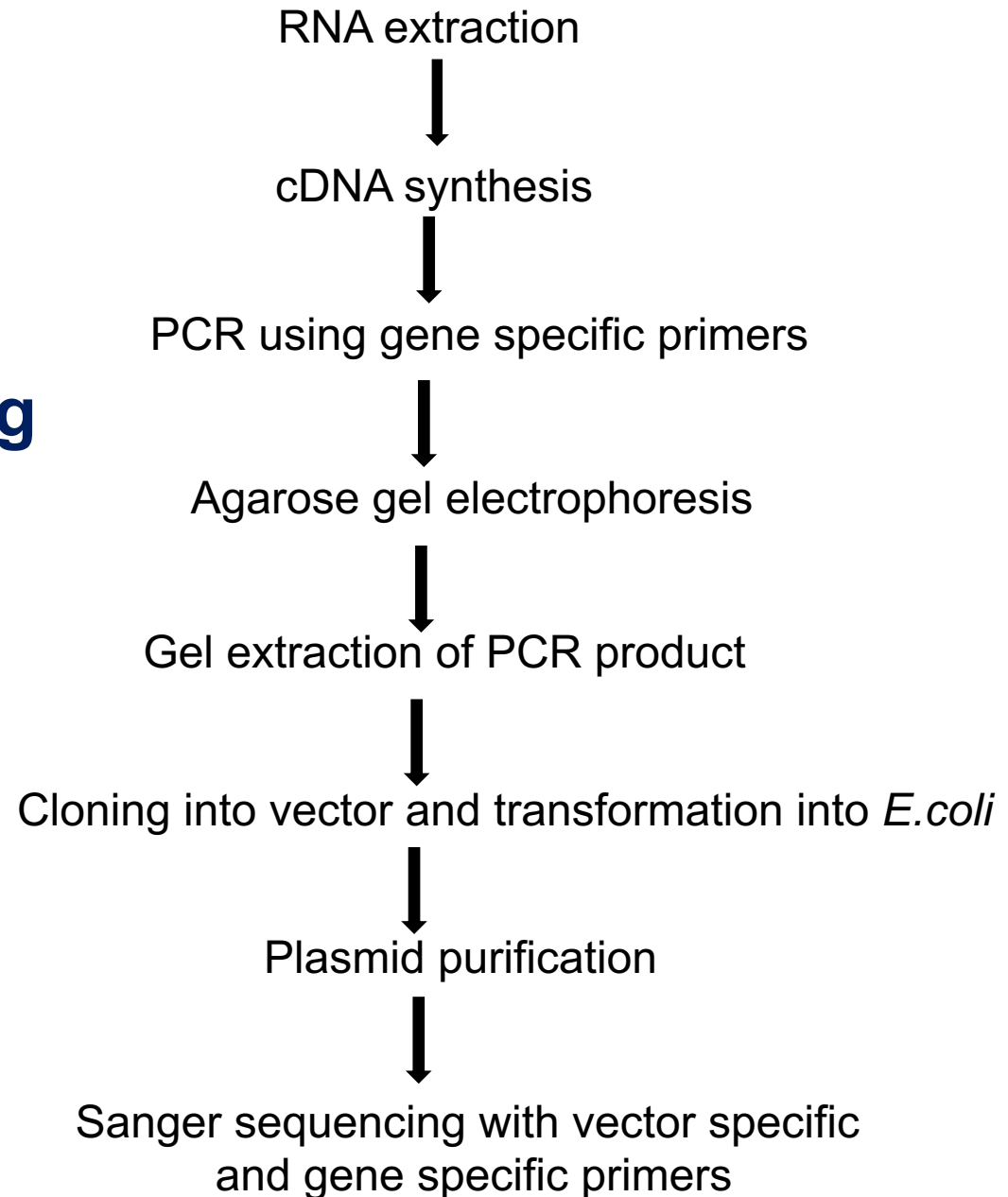


Plasmid purification



Sanger Sequencing with vector specific and gene specific primers

General workflow for cloning of a target gene from cDNA



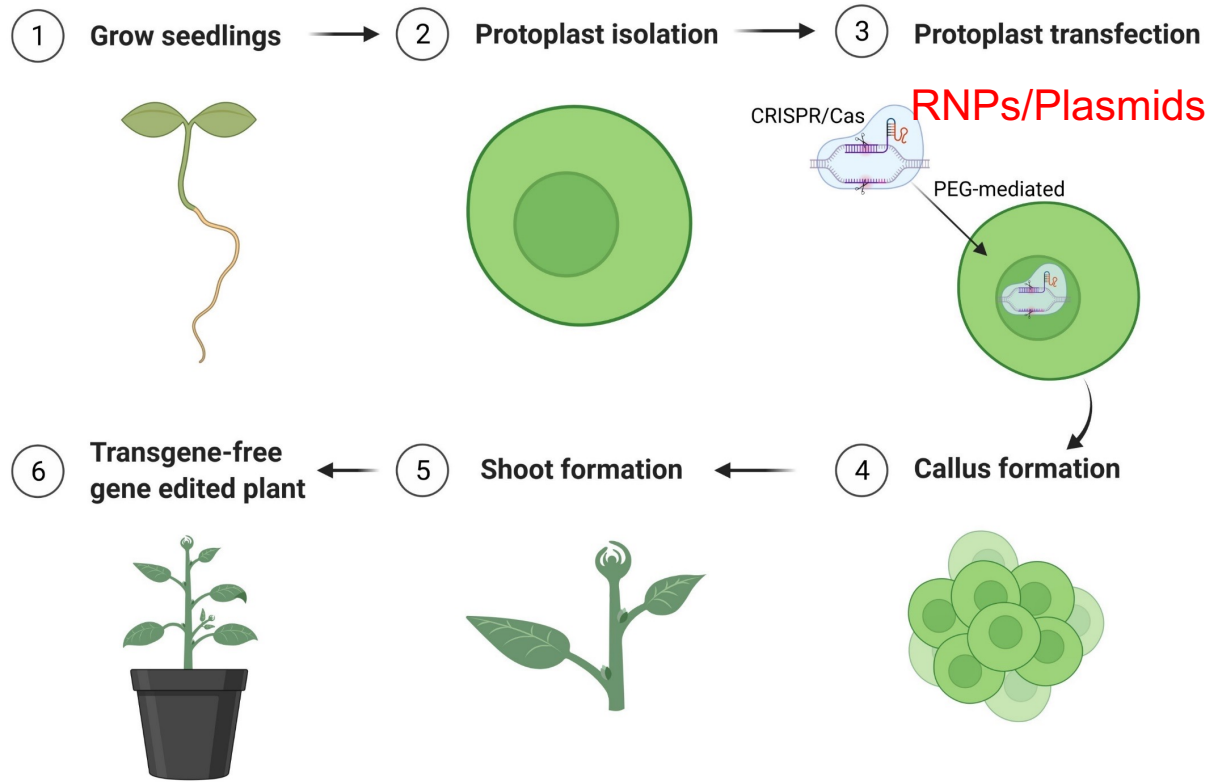
Nucleotide similarity of *GTR1* genes in *B. napus*

	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	

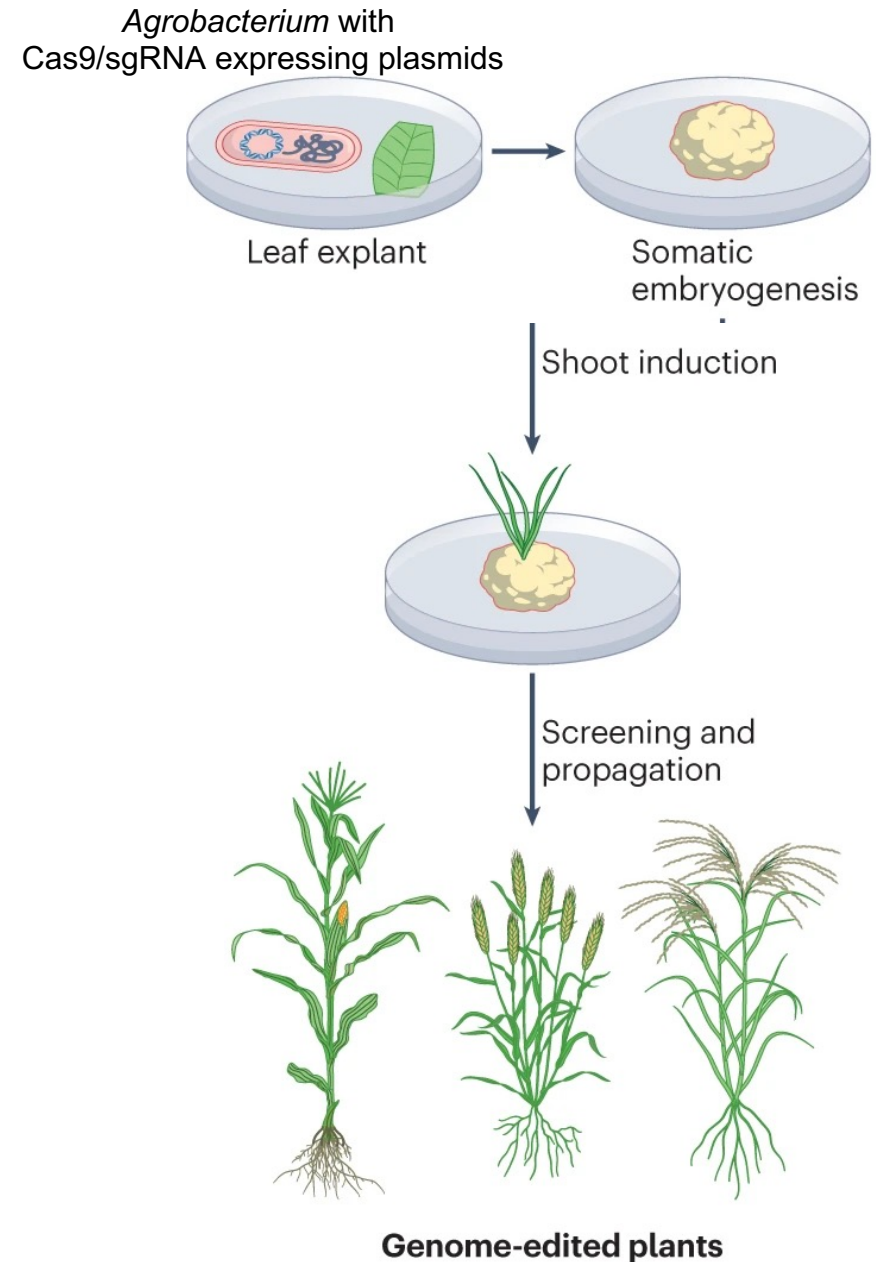
Protein identity of *GTR1* genes in *B. napus*

	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	

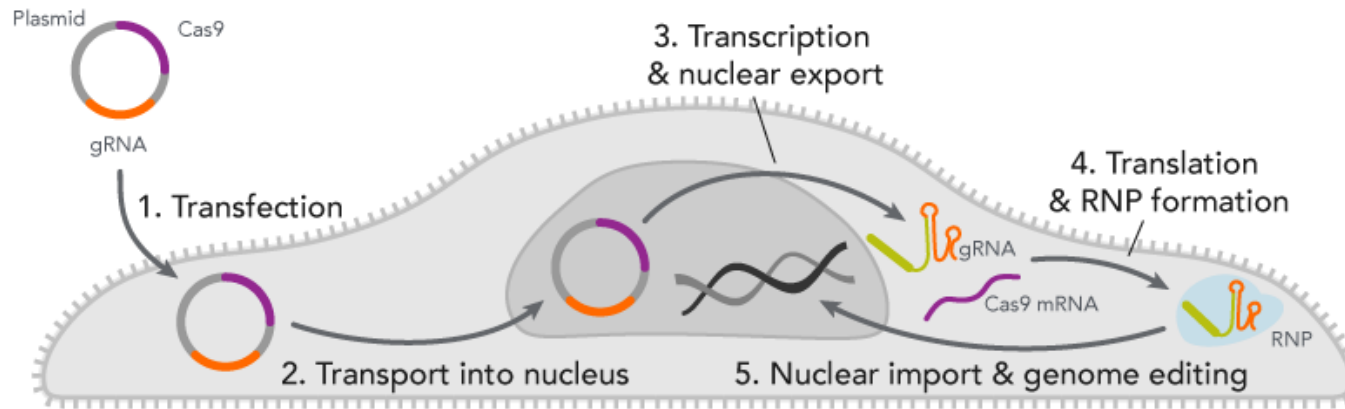
Transformation Techniques (Tissue culture dependent)



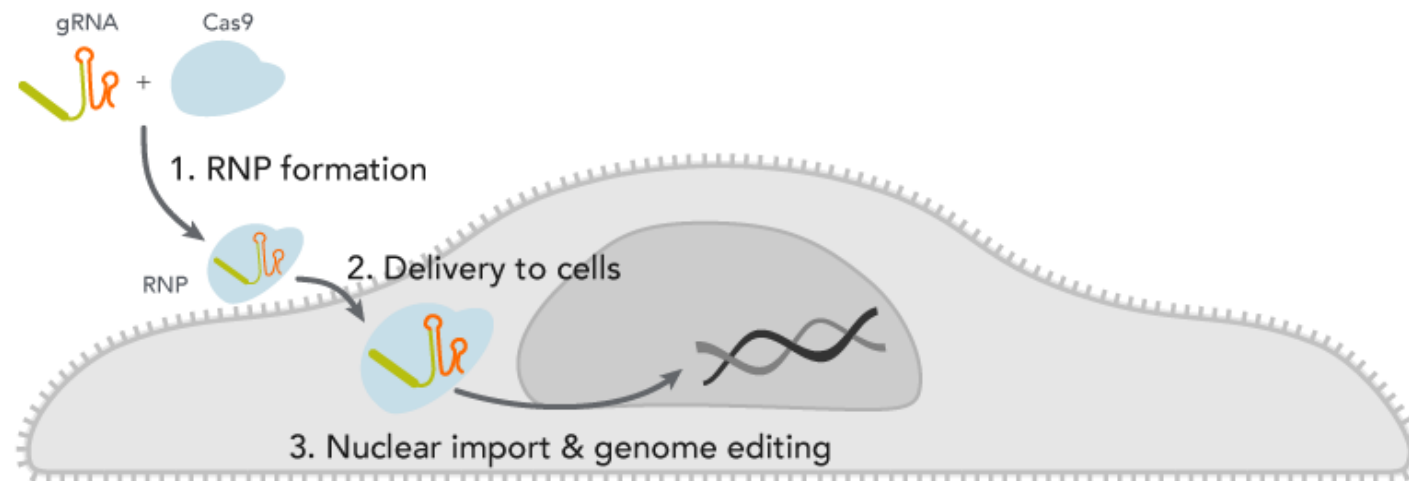
Genome editing using protoplasts
Homozygotic transgene-free mutants
at T0 generation



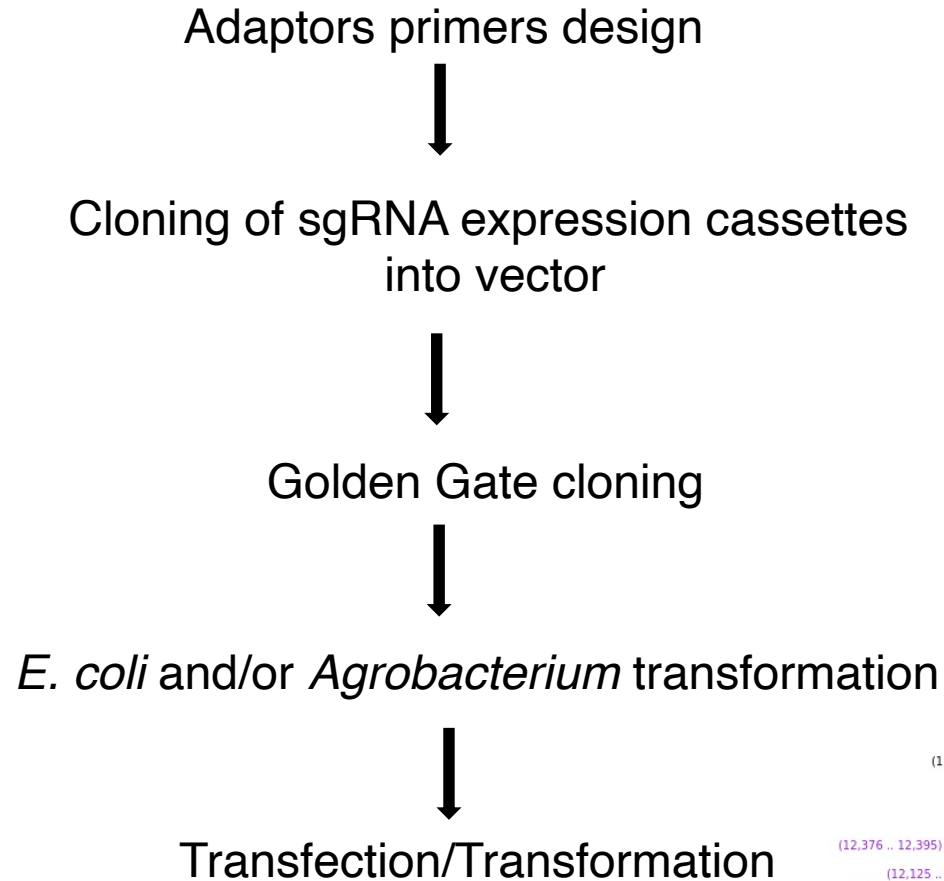
Plasmid-based editing



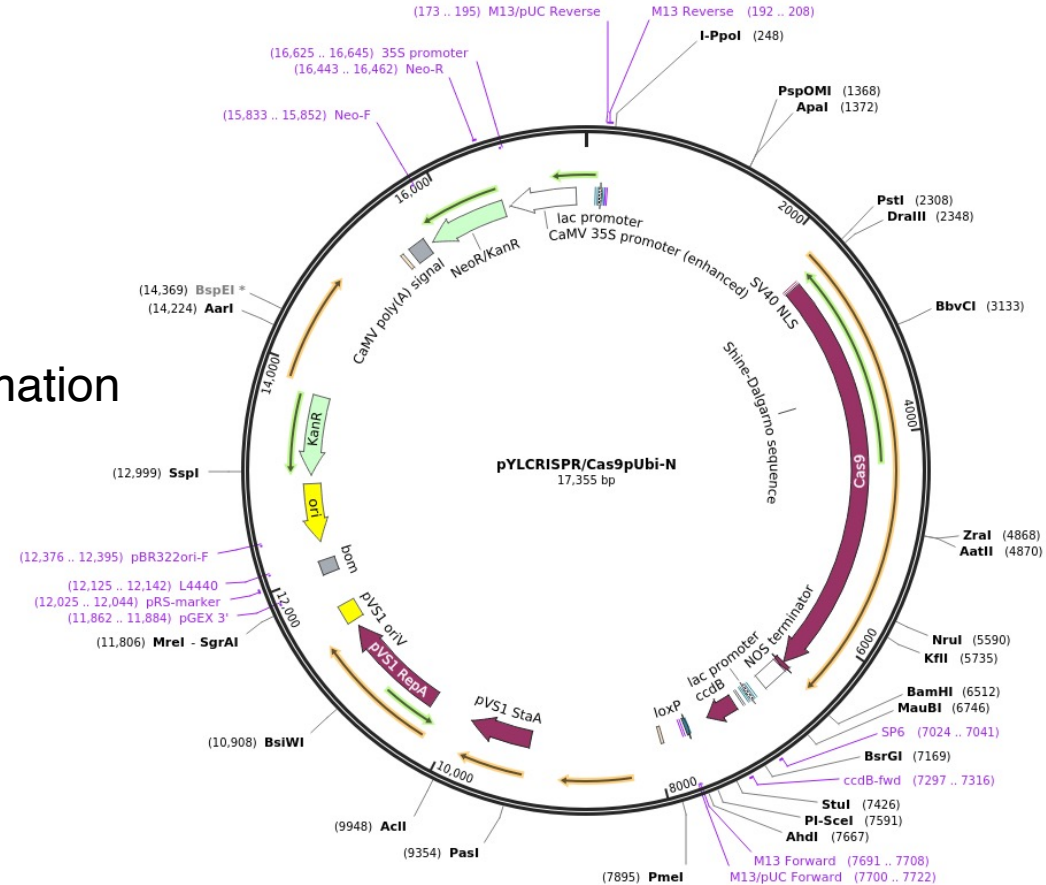
RNP-based editing



Plasmid-based editing



Vector Map

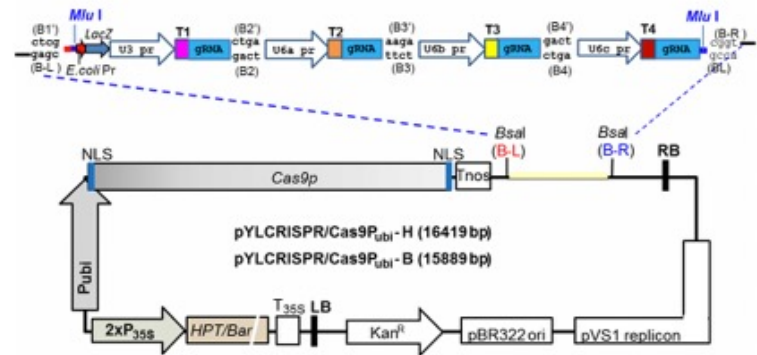


Adaptors primers design



primerDesign-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.



[Go to primerDesign-V tool](#)



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 ①	Primers (5'-3')	Tm
1	AATGAGACATTTGAGAAGAT	AtU3d	method1	AtU3dT1F: gtcaATGAGACATTTGAGAAGAT AtU3dT1R: aaacATCTTCTCAAATGCTCAT	58.2 56.4
2	GAATCAACAGTTTCTTCAAC	AtU3b	method1	AtU3bT2F: gtcaGAATCAACAGTTTCTTCAAC AtU3bT2R: aaacGTTGAAGAACTGTTGATTC	60.4 58.7

[Insert](#) [Delete](#) [Design](#)

<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

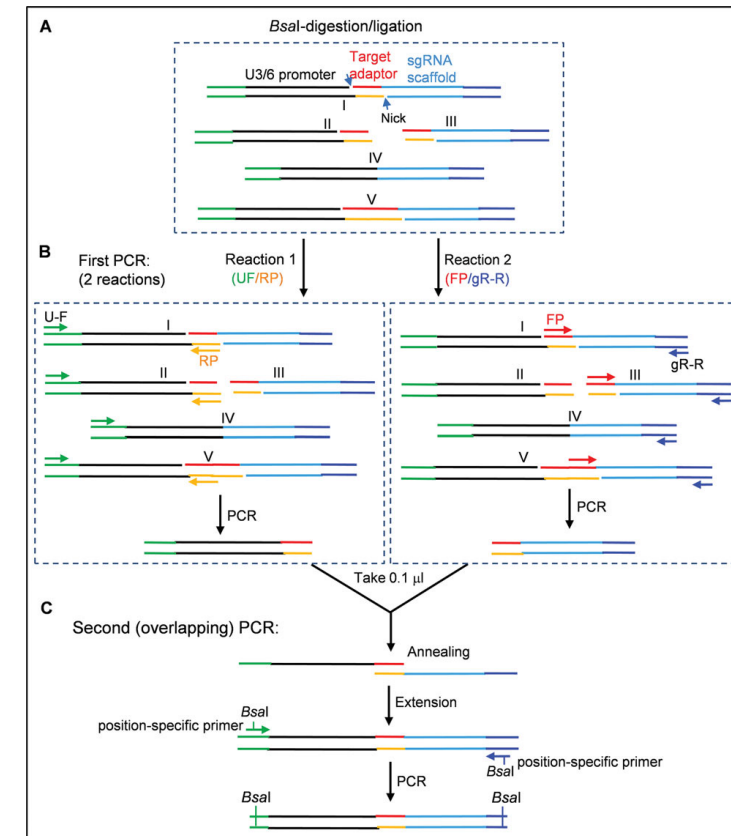
3 targets: LacZ-AtU3d—AtU3b—AtU6-1

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

gRNA Design



CRISPOR ([citation](#)) is a program that helps design, evaluate and clone guide sequences for the CRISPR/Cas9 system. [CRISPOR Manual](#)
July 2023: A faster server has arrived and should come online within 2-3 months. Python3 upgrade on hold, the ML models output different scores on Py3. [Full list of changes](#)

Step 1

Planning a lentiviral gene knockout screen? Use [CRISPOR Batch](#)

Sequence name (optional):

Enter a single genomic sequence, < 2300 bp, typically an exon

[Clear Box](#) - [Reset to default](#)

```
cttcctttgtcccaactctggcgcgccgcccctggcgccctaaggactggcgccggaagtggcaggcgggggcgacctcggtcaca  
gcgcccggtattctcgagctacacatgatgatgatcggcgccgctcgctgcgcacacggctccggcatgatgcgaaggccg  
gcttcggcgccgacgatgcccccgggccgcttccccctccatcgtggggcgcc
```

Step 2

Select a genome

We have 1029 genomes, but not yours? Search [NCBI assembly](#) and send a GCF_/GCA_ ID to [CRISPOR support](#).

Step 3

Select a Protospacer Adjacent Motif (PAM)

See [notes on enzymes](#) in the manual.

[SUBMIT](#)

CCTop - CRISPR/Cas9 target online predictor



CENTRE FOR
ORGANISMAL STUDIES

name:

☒ single query

☐ batch mode

query sequence: (plain nucleotide sequence, max 500 bases) (multi-)fasta file no file selected

be notified by email

PAM type :

Target selection

Off-target prediction

5' limitation
5' NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGG
3' limitation

max 4 MM
5' NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGG
max 2 MM core

target site length :

target site 5' limitation :

target site 3' limitation :

In vitro transcription

☒ T7 ☐ U6 ☐ Custom

fwd overhang:

rev overhang:

max. total mismatches :

☒ core length :

max. core mismatches :

species :

Petunia (Petunia axillaris)

Pisum sativum (pea)

Populus alba (sPta717 v2)

Populus tremula (sPta717 v2)

Populus trichocarpa (JGI v3.1)

Potato (Solanum tuberosum DM1-3 v4.04)



CRISPR MultiTargeter

Search for common and unique CRISPR guide RNA targets in multiple similar sequences

5' dinucleotide

Target length

PAM sequence orientation

☒ 5'

☐ 3'

Allow a mismatch in the first 8 nucleotides?

☒ Yes

☐ No

PAM sequence

☒ NGG (scoring is available*)

☐ Your own PAM sequence

*Scoring is available for 20-nt type II sgRNAs. See [Dönnch et al., 2014](#) for details. High scores may indicate more potent sgRNAs. However, some potent sgRNAs have scores < 0.1 and a number of successful sgRNAs had scores between 0.1 and 0.2. Therefore, potential experimental utility and subsequent experimental verification should guide the choice of sgRNAs.

Off-target analysis parameters:

CT-Scan: No restrictions on PAM sequence or target length. Adjust the target rule on the submission form.

Cas-Offfinder: Length should be 20bp for SpCas9 (PAM = "NGG"), 18bp for StCas9 (PAM = "NNAGAAM"), and 24bp for NmCas9 (PAM = "NNNNMMTTT").

Input sequence(s)*



CRISPR MultiTargeter

Search for common and unique CRISPR guide RNA targets in multiple similar sequences

5' dinucleotide

Target length

PAM sequence orientation

☒ 5'

☐ 3'

Allow a mismatch in the first 8 nucleotides?

☒ Yes

☐ No

PAM sequence

☒ NGG (scoring is available*)

☐ Your own PAM sequence

*Scoring is available for 20-nt type II sgRNAs. See [Dönnch et al., 2014](#) for details. High scores may indicate more potent sgRNAs. However, some potent sgRNAs have scores < 0.1 and a number of successful sgRNAs had scores between 0.1 and 0.2. Therefore, potential experimental utility and subsequent experimental verification should guide the choice of sgRNAs.

Off-target analysis parameters:

CT-Scan: No restrictions on PAM sequence or target length. Adjust the target rule on the submission form.

Cas-Offfinder: Length should be 20bp for SpCas9 (PAM = "NGG"), 18bp for StCas9 (PAM = "NNAGAAM"), and 24bp for NmCas9 (PAM = "NNNNMMTTT").

Input sequence(s)*

gRNAs targeting Multisequence

https://multicrispr.net/multalign_input.html

Supported by:



Off Target Prediction

Cas-OFFinder

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-OFFinder: 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-OFFinder 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'-NNNNGMTT-3' (M = A or C)
- ☐ SaCas9 from Staphylococcus aureus: 5'-NNGRRT-3' (R=A or G)
- ☐ CjCas9 from Campylobacter jejuni: 5'-NNNVRYAC-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!**

Mismatch
Number
(eq or less than)

DNA Bulge Size
(eq or less than)



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

PAM type:

* Or define your own PAM:

PAM sequence:

PAM position:

Guide length (nt):

Target (reference) genome:

Query

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

ID	Target sequence (5'-3')	PAM
Target sequence 1:	<input type="text"/>	<input type="text"/>

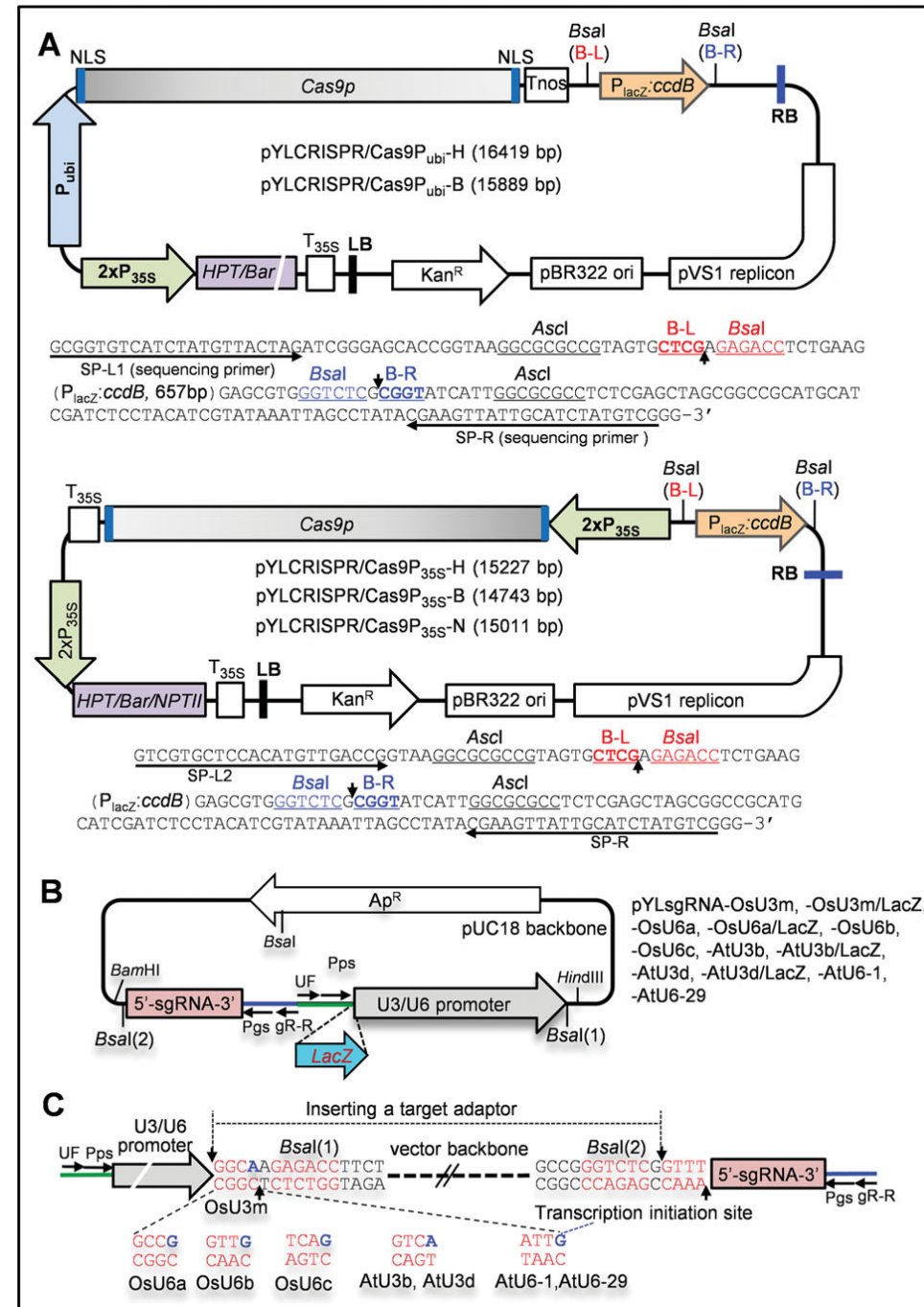
Target Genome

Organism Type

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/Ensplant26) - 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 IKGC) - 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



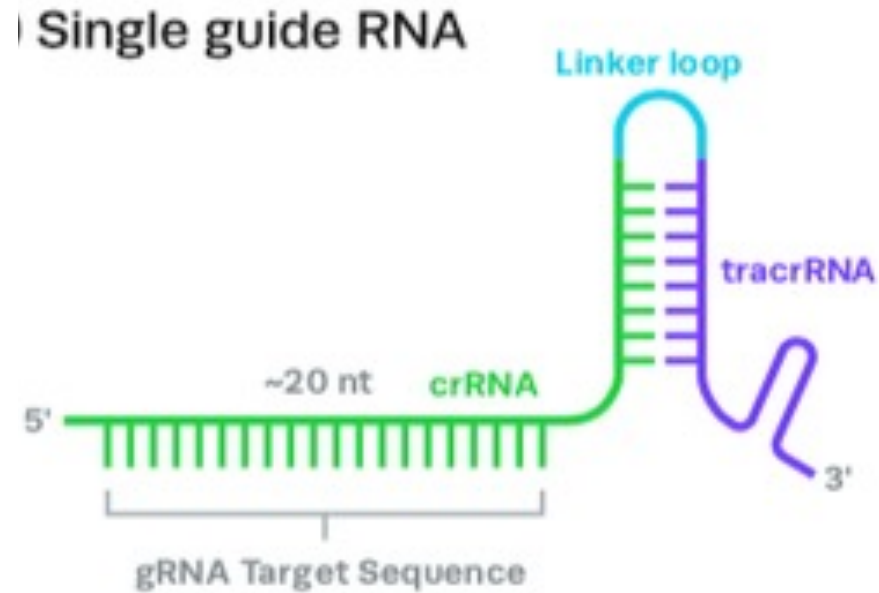
sgRNA production



RNP assembly



Transfection to protoplasts



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

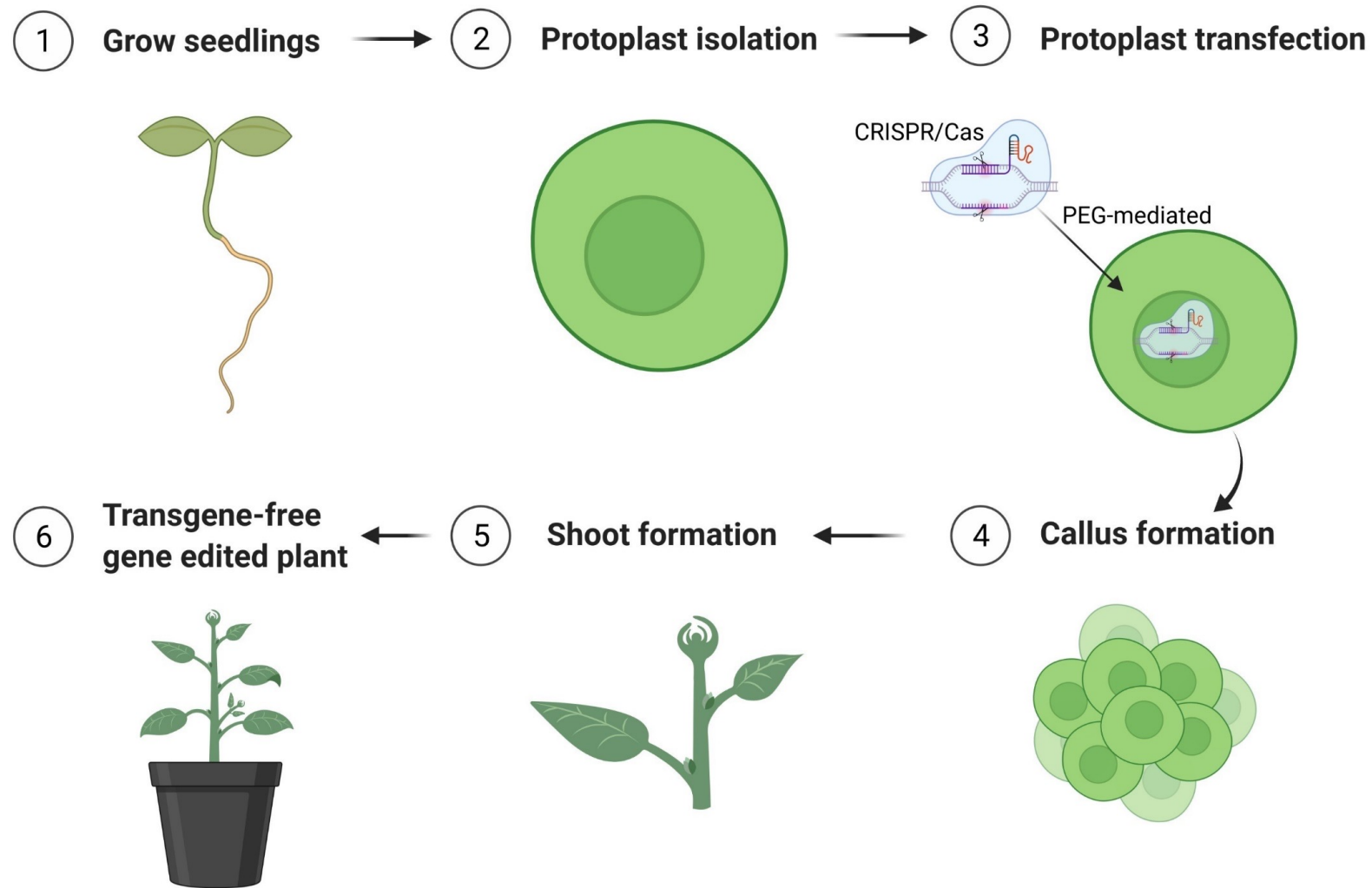
Advantages

- 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

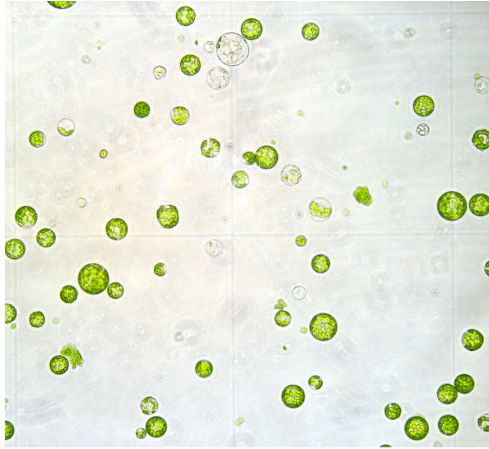
Disadvantages

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

Workflow for genome editing using protoplasts



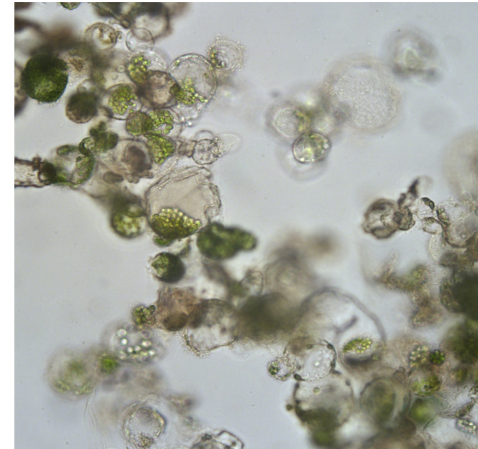
PEG-mediated protoplast transfection/regeneration



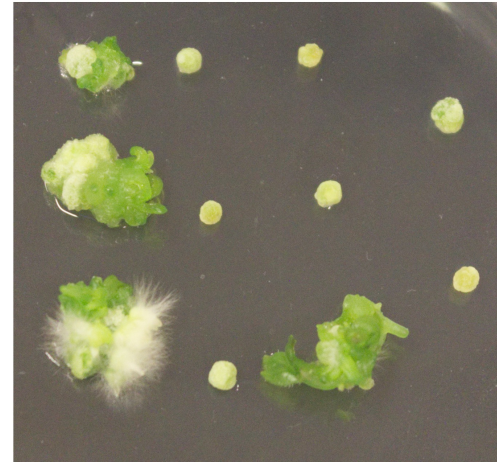
Freshly isolated
protoplasts



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](#)

[Start TIDE](#)



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 TIDER](#)

SYNTHGO
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) Multi-guide
17-23 nt RNA sequences without PAM

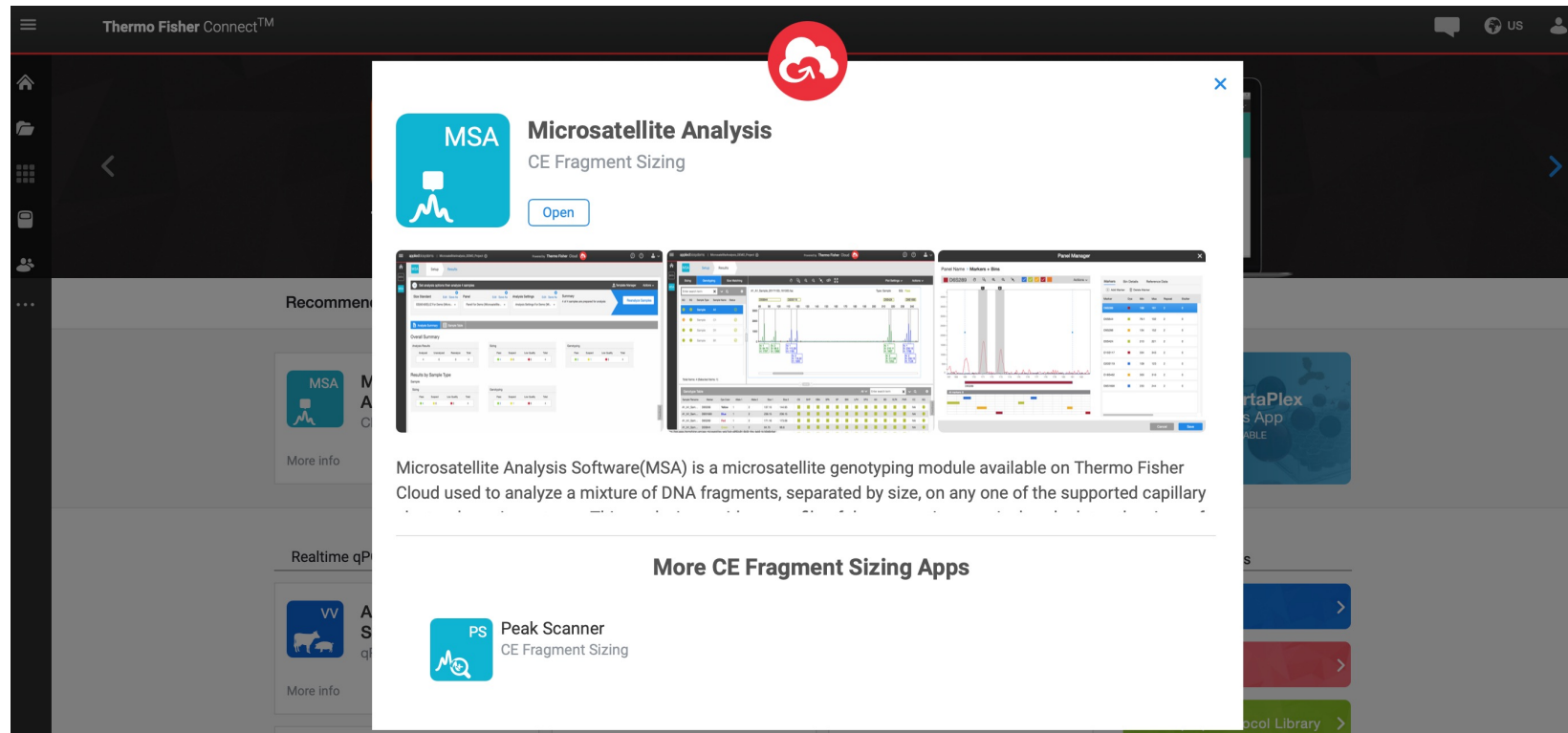
Donor Sequence (Optional) Knockin
16-300 nt DNA sequence with homology arms. Homology arm must have at least 15 bp of alignment.

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

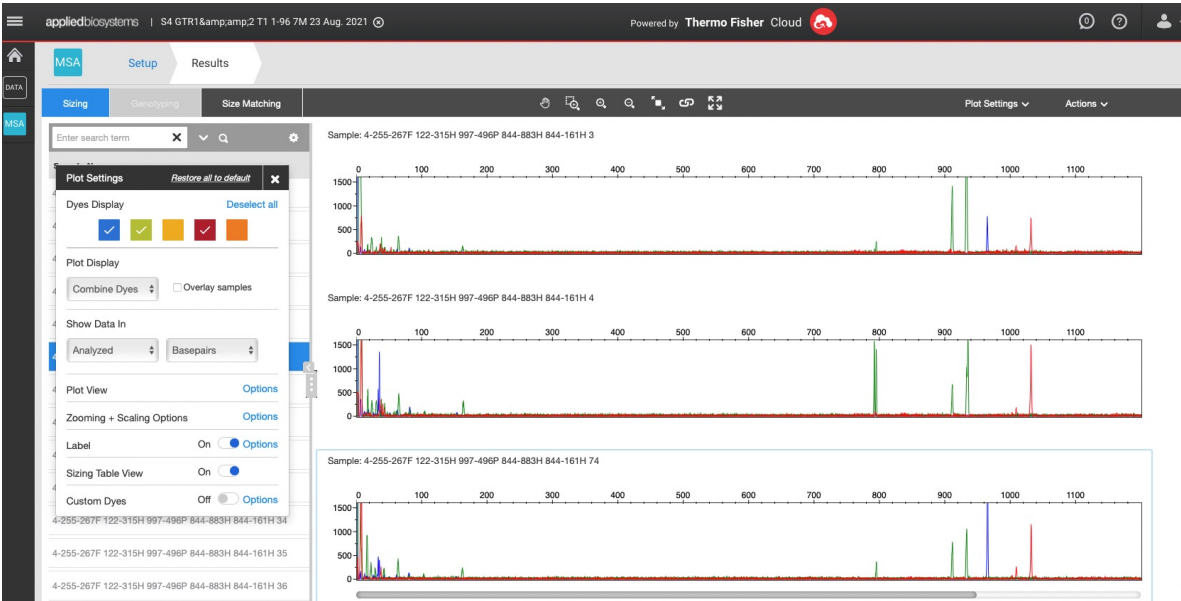
- ❖ 3500 Genetic analyzer
- ❖ PCR with one of the primer attached Florescent dyes
- ❖ 6 different dyes at the same time
- ❖ GeneMapper™ analysis
- ❖ Thermo Fisher Connect™



<https://apps.thermofisher.com/apps/spa/#/dashboard>

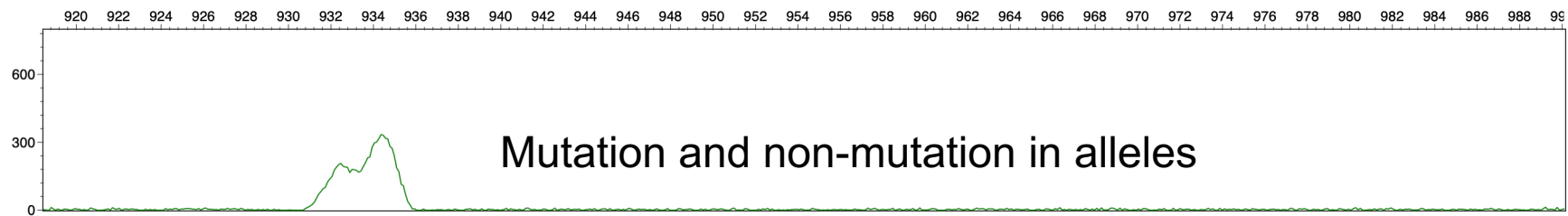
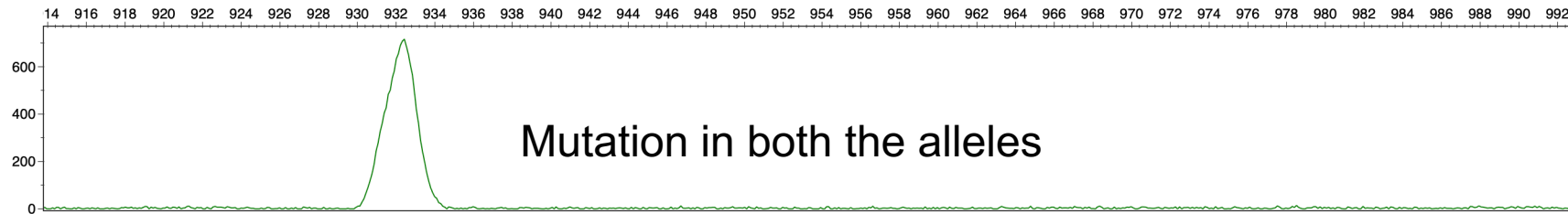
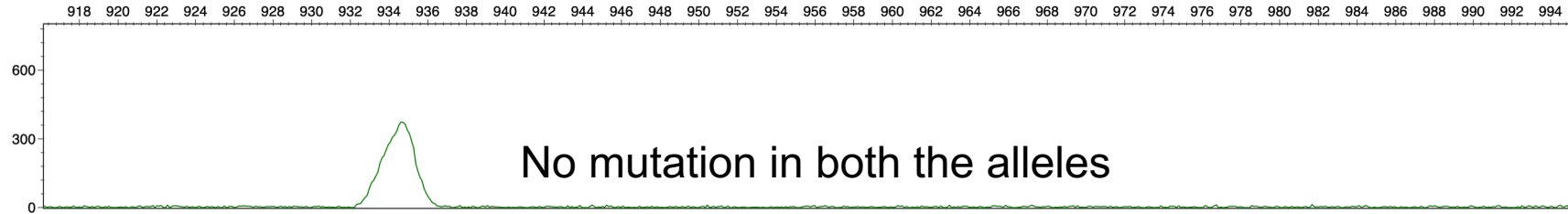
High Resolution Fragment Analysis

Fragment analysis using Thermo Fisher Connect™

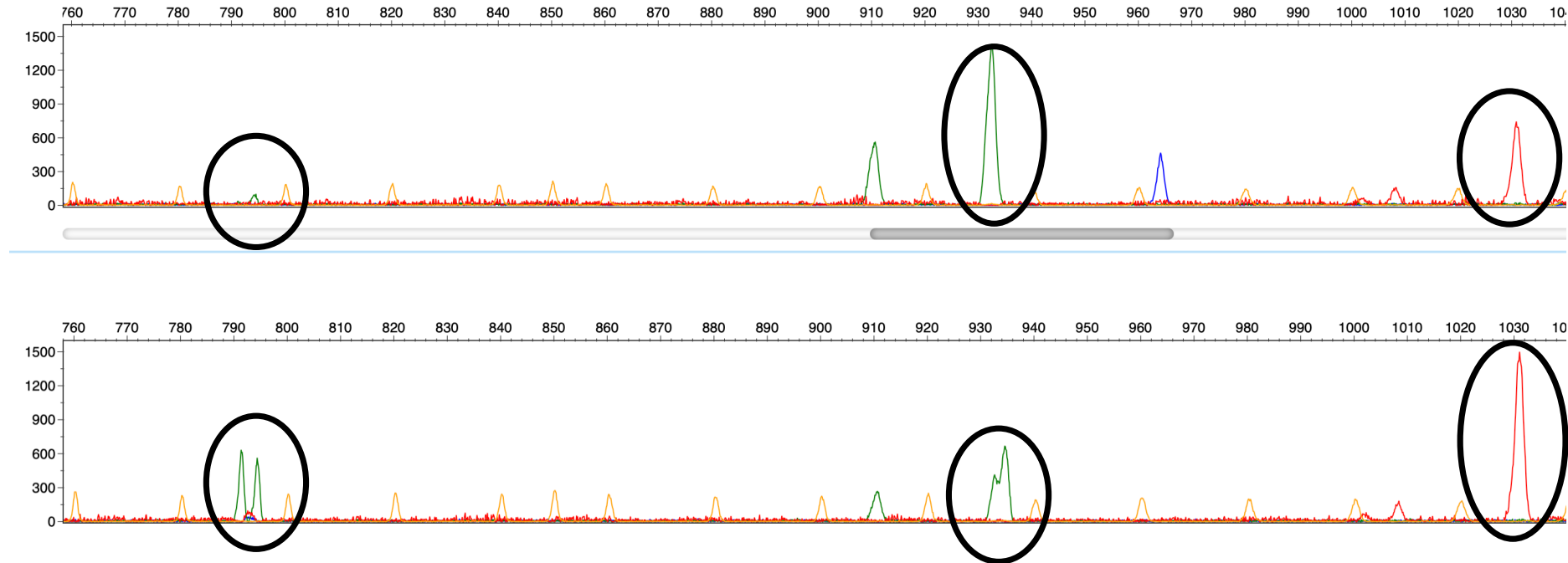


Multiple Samples									
Edit Peaks ▾ All Peaks ▾ Enter search term									
Peak Overview	Dye Color	Sample Name	Size	Height	Area (Data Point)	Area (Base Pairs)	Begin Point (Base Pa...	End Point (Base Pairs)	Show/Hide Columns
403 Peaks	Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	993.88	74.00	350.00	38.93	993.10	995.21	<input type="checkbox"/> Sample Filename <input checked="" type="checkbox"/> Dye Color <input type="checkbox"/> Dye, Sample Peak <input checked="" type="checkbox"/> Sample Name <input checked="" type="checkbox"/> Size <input checked="" type="checkbox"/> Height <input checked="" type="checkbox"/> Area (Data Point) <input checked="" type="checkbox"/> Area (Base Pairs) <input type="checkbox"/> Data Point <input type="checkbox"/> Data Point Restore to default Apply
	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	
20 YELLOW	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	168.00	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	
204 ORANGE	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	
145 RED	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	
27 GREEN	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	
7 BLUE									

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

NGS-Illumina-Next Generation Sequencing Technologies

Amplicon sequencing Analysis



CRISPResso2

Analysis of genome editing outcomes from deep sequencing data

CRISPResso2

geneious
prime

Cas-Analyzer

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

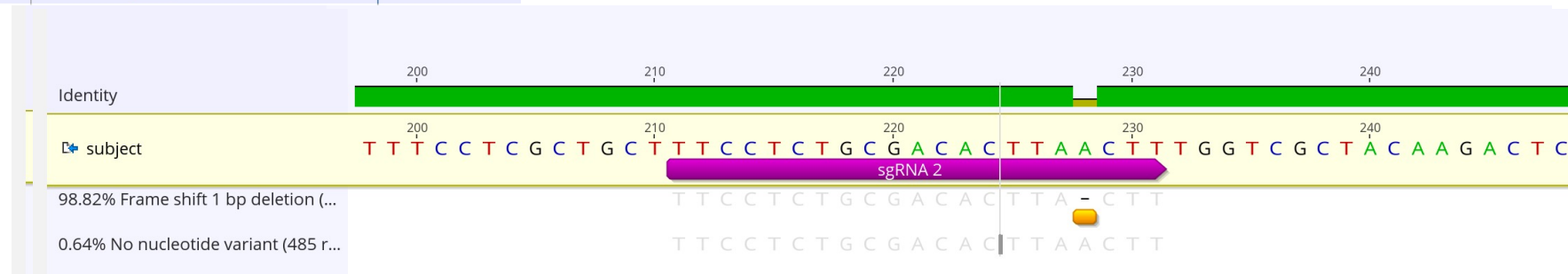
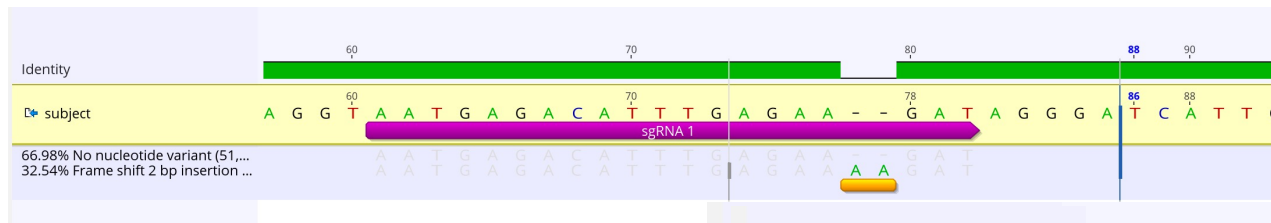
Thanks to the improvements in the newest JavaScript engines in the most recent web browsers, the JavaScript based internal algorithm of Cas-Analyzer completely runs on the client-side so that large amounts of sequencing data do not need to be uploaded to the server. Currently, Cas-Analyzer supports various single nucleases (SpCas9, StCas9, NmCas9, SaCas9, CjCas9, and AsCpf1/LbCpf1) and paired nucleases (ZFNs, TALENs, Cas9 nickases, and dCas9-FokI 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: <input type="text" value="Paired-end reads"/>	
Read 1 (fastq or gzipped fastq): <input type="button" value="Choose File"/> no file selected	Read 2 (fastq or gzipped fastq): <input type="button" value="Choose File"/> no file selected
Basic Information	
Full reference sequence (5' to 3'): <input type="text"/>	
Nuclease Type: <input type="text" value="Single nuclease"/>	
Select Nuclease: <input type="text"/>	
Analysis Parameters	
Comparison range (R) [?] <input type="text" value="70"/> or use both ends	
Minimum frequency (n) [?] <input type="text" value="1"/>	
<input checked="" type="checkbox"/> (Optional) WT marker (r) [?] <input type="text" value="5"/>	



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?

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