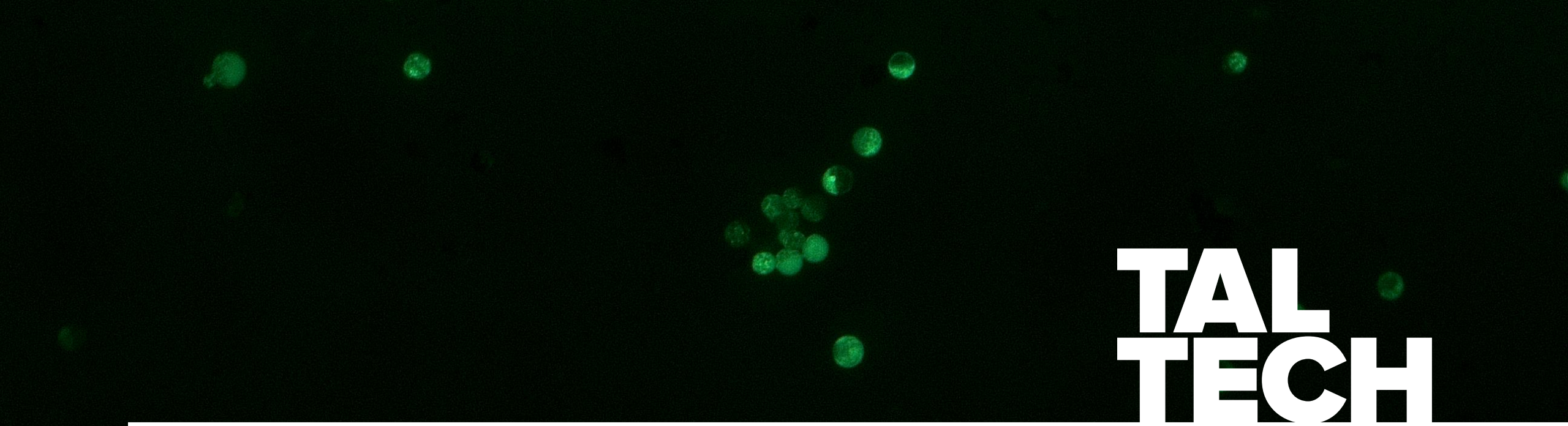


GENOME EDITING IN PERENNIAL RYEGRASS PROTOPLASTS

Ferenz Sustek Sánchez
Institute of Chemistry and Biotechnology
Tallinn University of Technology

Iceland Liechtenstein Norway grants

EDIT GRASS 4FOOD

05.04.24

PERENNIAL RYEGRASS

- *Lolium perenne* L. is
  - Important temperate grass used for forage and turf
  - The most widely cultivated forage grass in Europe
- However, it doesn't grow well under
  - Drought
  - Freezing

→ Limits its cultivation in Northern regions

&

Concern due to climate change

TAL TECH

GENETICS AND REPRODUCTION OF *Lolium perenne*

- Wind pollinated
- Obligate outcrossing species
  - Self-incompatible gametophyte
  - Highly heterogenous genome

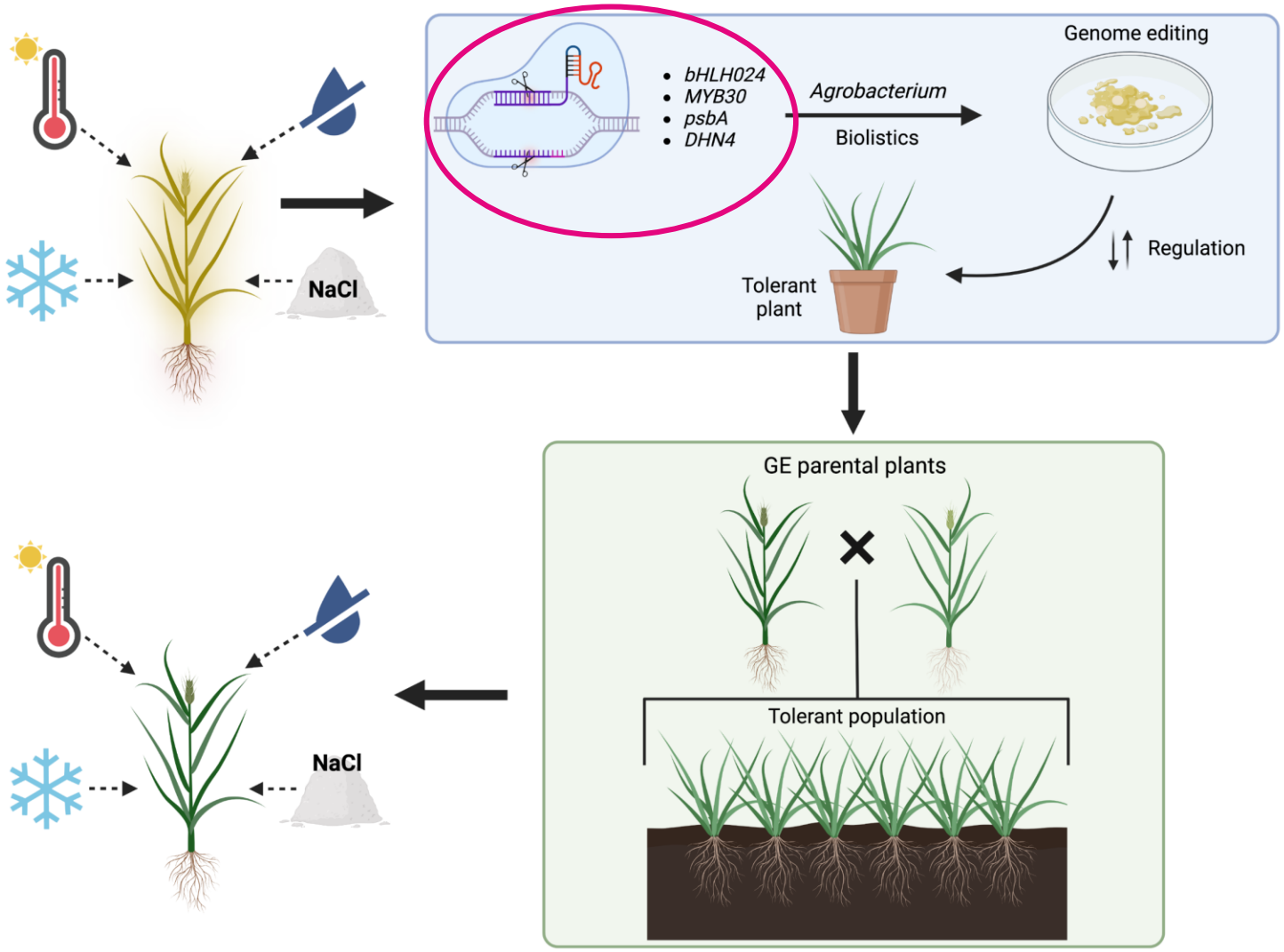
- Selective and traditional breeding
  - Not the best option for generation of abiotic tolerant lines

Genome Editing

Using CRISPR-Cas to create tolerant plants

TAL TECH

GENOME EDITING WITH CRISPR-CAS SYSTEMS



From Sustek-Sanchez et al., 2023

DESIGN AND SELECTION OF gRNAs

- Mostly using bioinformatic tools
- Provide scores for
  - Specificity
  - Activity/Efficiency
- The predicted activity is based on
  - The nucleotides of the guide
  - Data sets of previous experiments

- Bottleneck
  - Predicted efficiency doesn't always correlate to the real activity of the guide

- Test activity of guides in vivo
  - Screen and select the best performing gRNAs

TAL TECH

USING PROTOPLASTS TO SELECT gRNAs

- Protoplasts are plant cells without a cell wall
- Used in multiple cellular, molecular and genetic studies
- Can be isolated by millions
  - Perfect for screening experiments

- Used for selection of gRNAs in plants
  - Arabidopsis (*Arabidopsis thaliana*)
  - Wheat (*Triticum aestivum*)
  - Rice (*Oryza sativa*)

TAL TECH

Lolium perenne PROTOPLASTS ISOLATION

- Different articles describe the isolation of perennial ryegrass protoplasts
- We tested to of the most recent ones
  - "An efficient protocol for perennial ryegrass mesophyll protoplast isolation and transformation, and its application on interaction study between LpNOL and LpNYC1" Yu et al. 2017
    - We did not manage to get the same high number of isolated cells
  - "Genetic Transformation of Protoplasts Isolated from Leaves of Lolium temulentum and Lolium perenne" Davis et al. 2020
    - We could not get a protoplast suspension with low amount of debris
- Therefore, we decided to establish a protocol based on these two publications

TAL TECH

DEVELOPING A PROTOPLAST ISOLATION METHOD

- Highly reproducible and with consistent high yields
  - Around 1 x 10^6 cells per mL of suspension
- Low amount of debris
- Testing different variables

Mannitol pretreatment (Molar concentration)	Cellulase concentration (w/v)	Enzymatic treatment length	Vacuum infiltration (KPa)
• 0.2 M • 0.3 M • 0.5 M • 0.6 M	• 1.5% • 2% • 2.5% • 3%	• 8 hours • 12 hours • 16 hours • 20 hours	• 0 • 71

TAL TECH

DROUGHT RESISTANCE THROUGH CRISPR-CAS KNOCKOUT

- Targeting gene *LpCBP20*
- Negatively regulates the synthesis of cuticular waxes
- Knocking out *cbp20* in barley produced drought resistant plants (Daszkowska-Golec *et al*. 2020)

TAL TECH

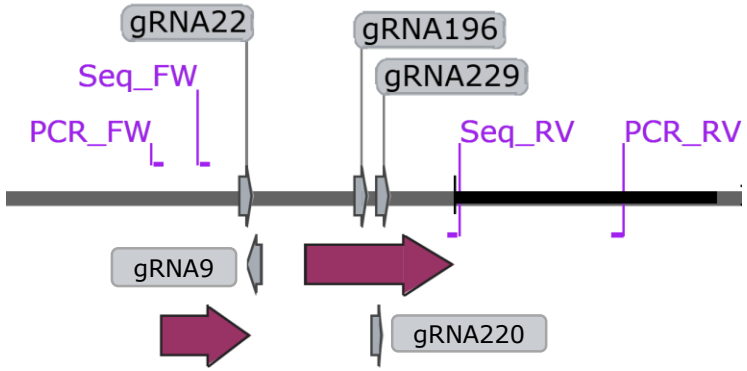
EXON 3’-UTR

CBP20
3409 bp

TRANSFORMING PROTOPLASTS WITH DIFFERENT VECTORS

- Using a plasmid with one single gRNA
  - EGFP cassette
- 3 different vectors targeting the second exon of CBP20
  - p196
  - p220
  - p229

- Using a plasmid with 5 different gRNAs
  - ZsGreen cassette
- One vector with
  - 2 gRNAs targeting the first exon
  - 3 gRNAs targeting the second exon



LpCBP20

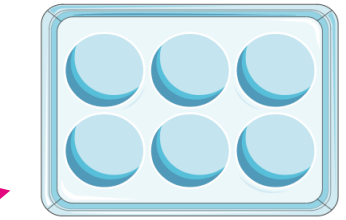
TAL TECH

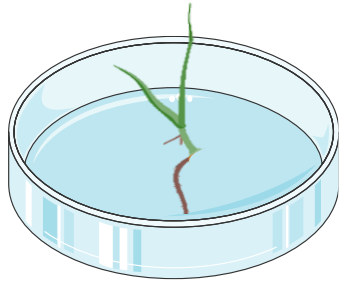
PLANT MATERIAL

Seeds from cultivar "VEJA"

Surface sterilization

Germination in liquid media: **½ MS** with 3% **sucrose**



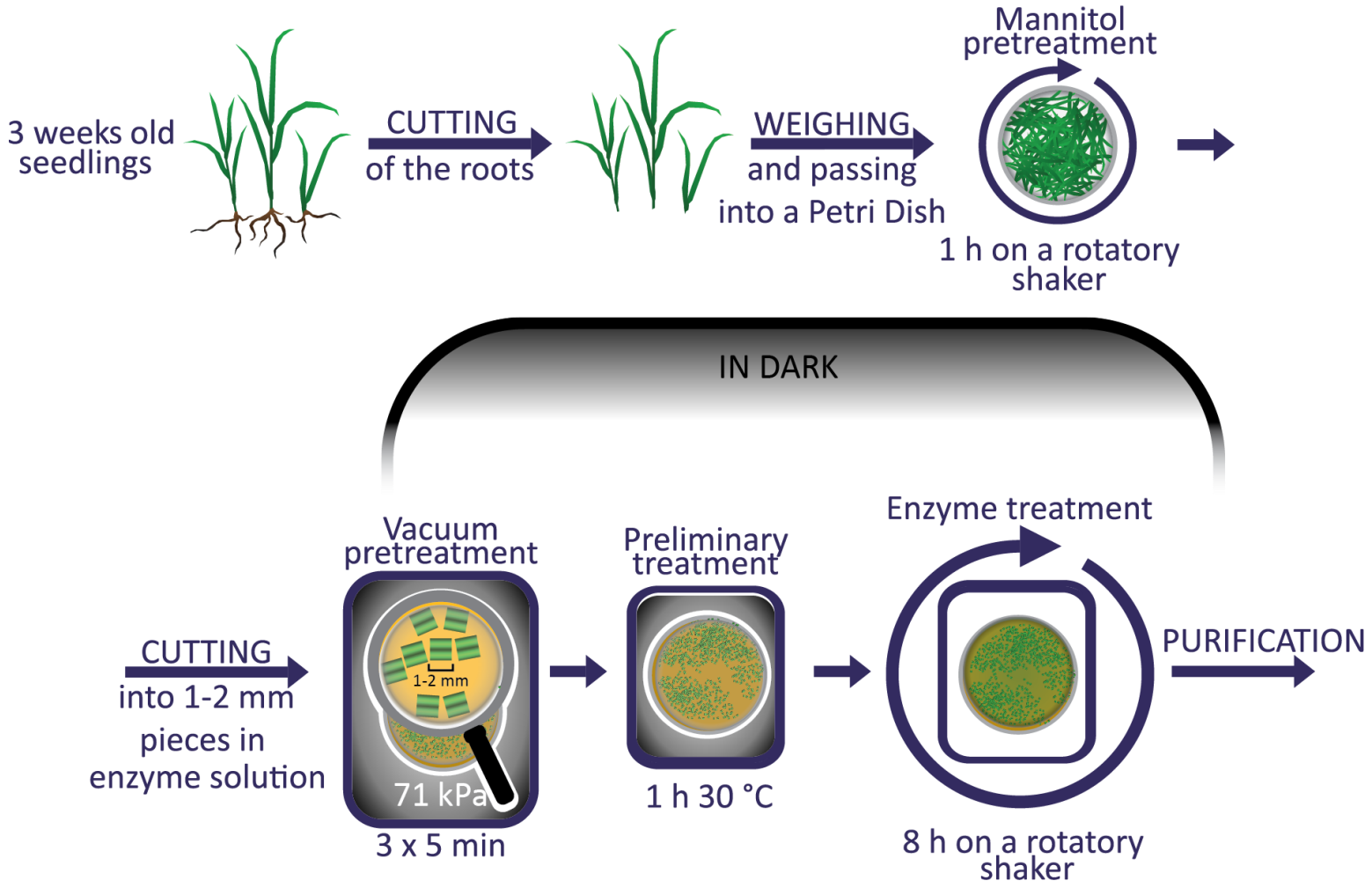


Propagation/Shoot generation: media with **BAP**

- 16 hours of photoperiod
- 21 °C
- 60% relative humidity

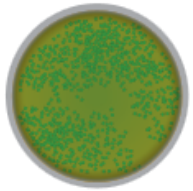
TAL TECH

PROTOPLASTS ISOLATION



TAL TECH

PROTOPLASTS PURIFICATION



WASHING & FILTERING

CENTRIFUGING
100 rcf 11 °C
5 min

RESUSPENSION

LAYERING
on 21% sucrose

CENTRIFUGING
100 rcf 11 °C
10 min

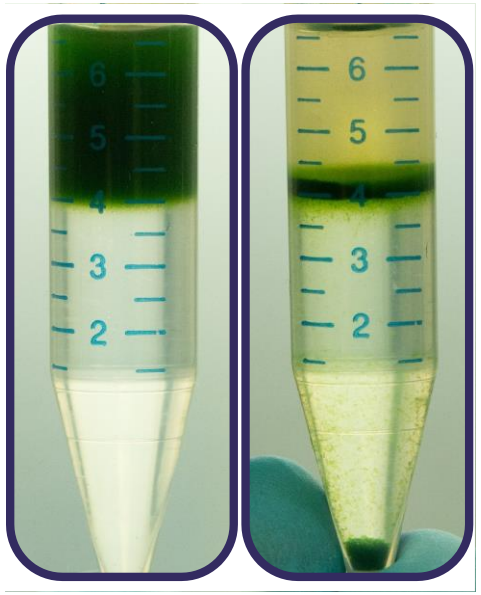
PASSING

ESTIMATION OF VIABILITY

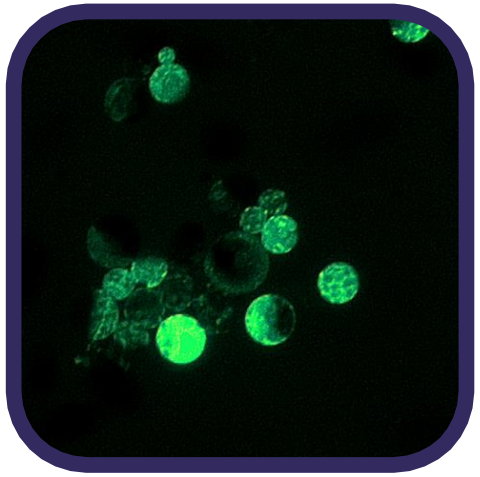
Settling
4 °C
overnight

PASSING

TRANSFORMATION



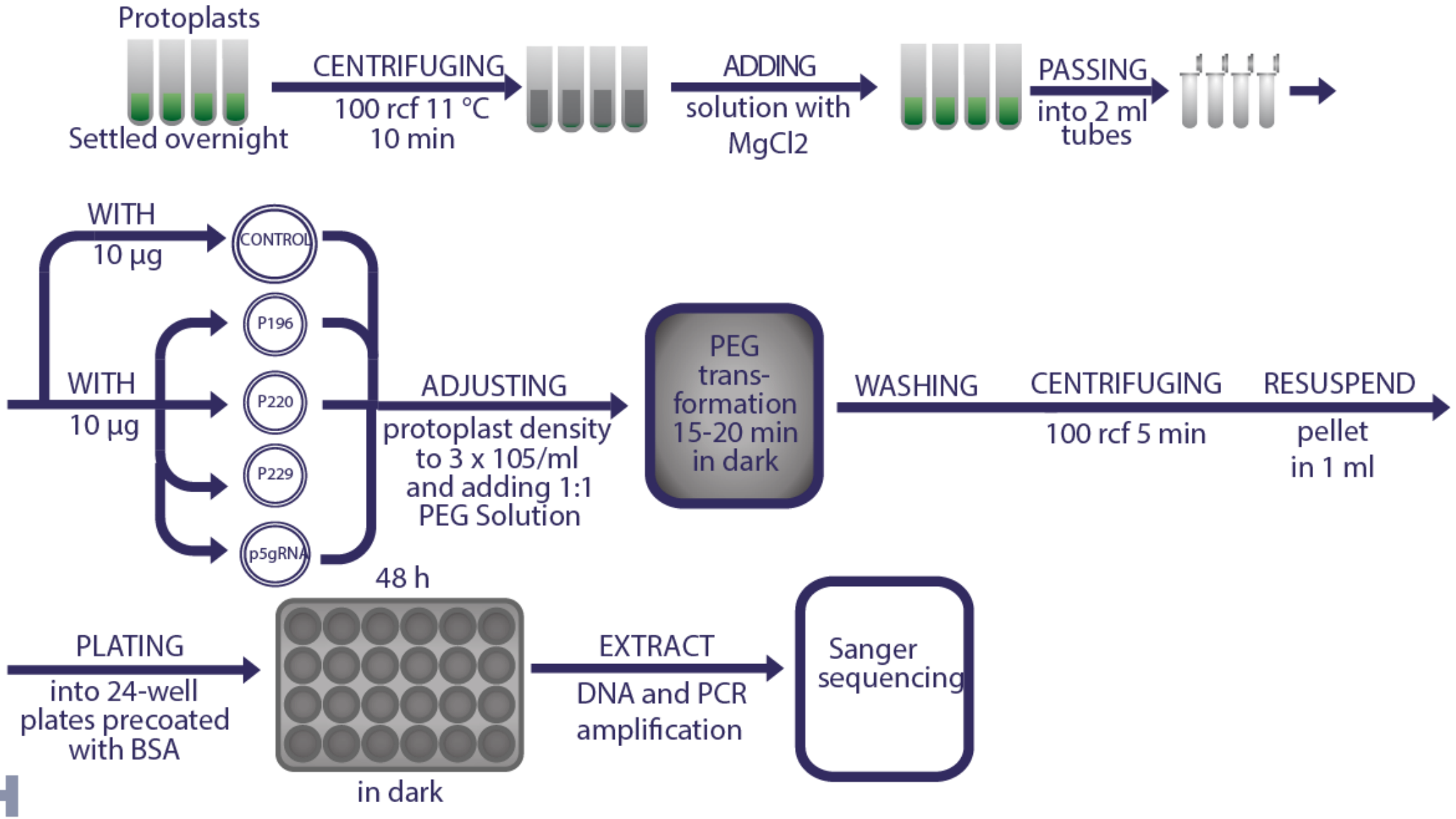
Fluorescein Diacetate



FDA

TAL TECH

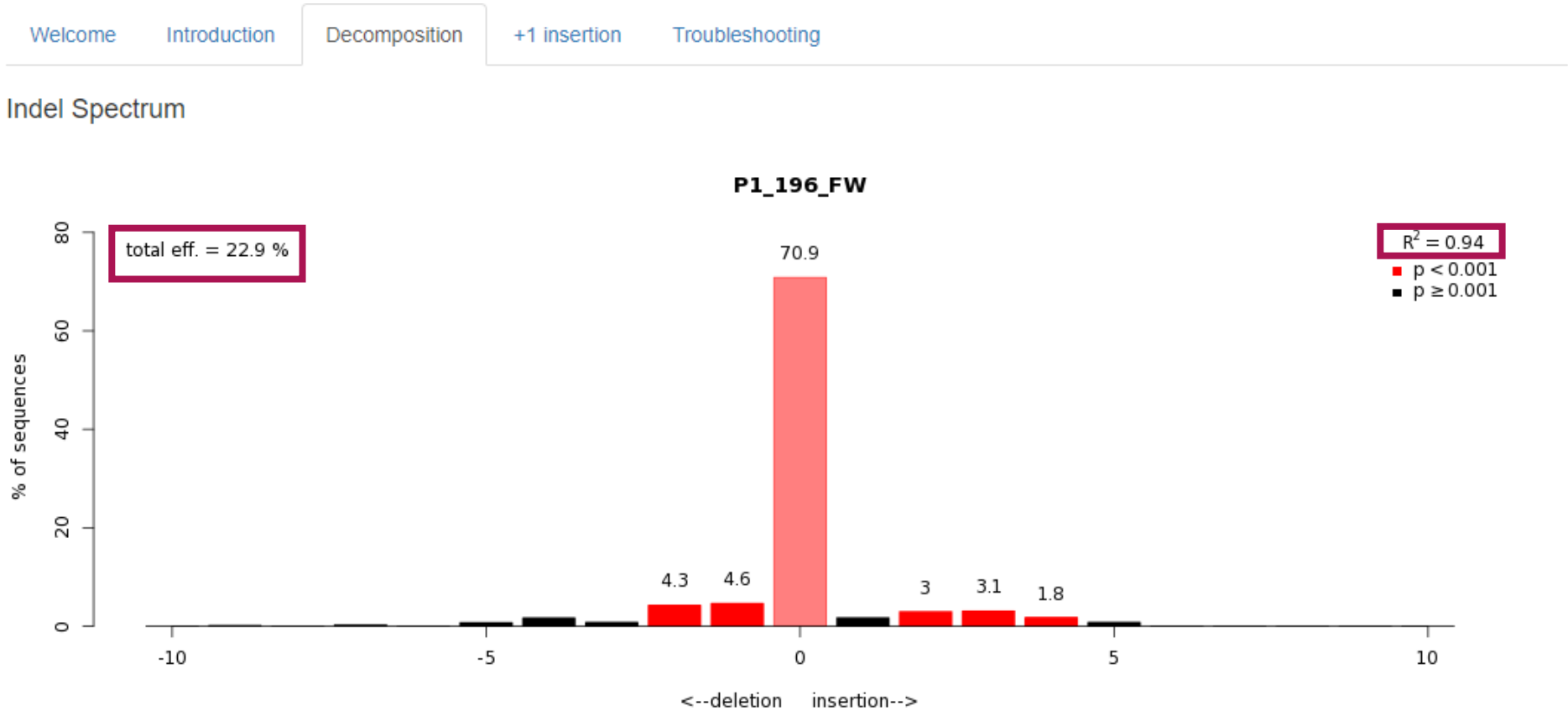
PROTOPLASTS TRANSFORMATION



TAL TECH

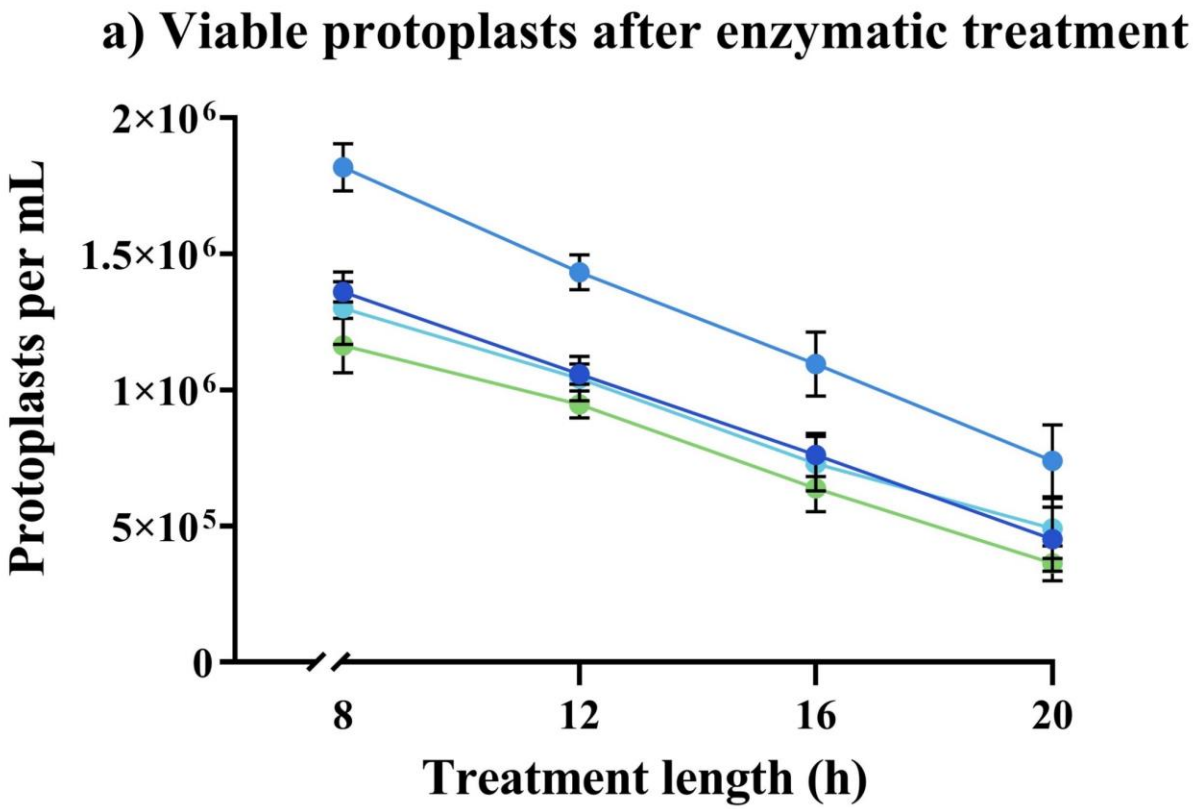
EDITING EFFICIENCY

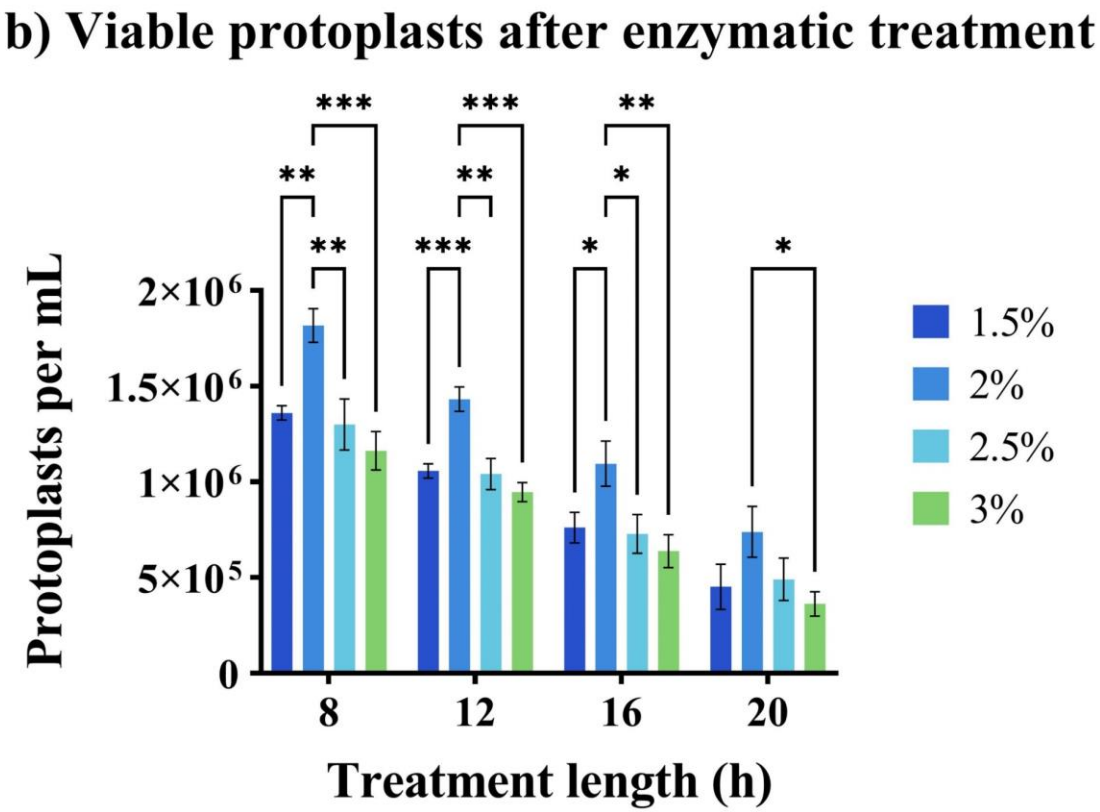
- Using Sanger sequencing trace data
- Analysis by decomposition using TIDE
- Compares non-transformed and transformed sequences
- Provides data showing the frequency of indels present in the samples



RESULTS

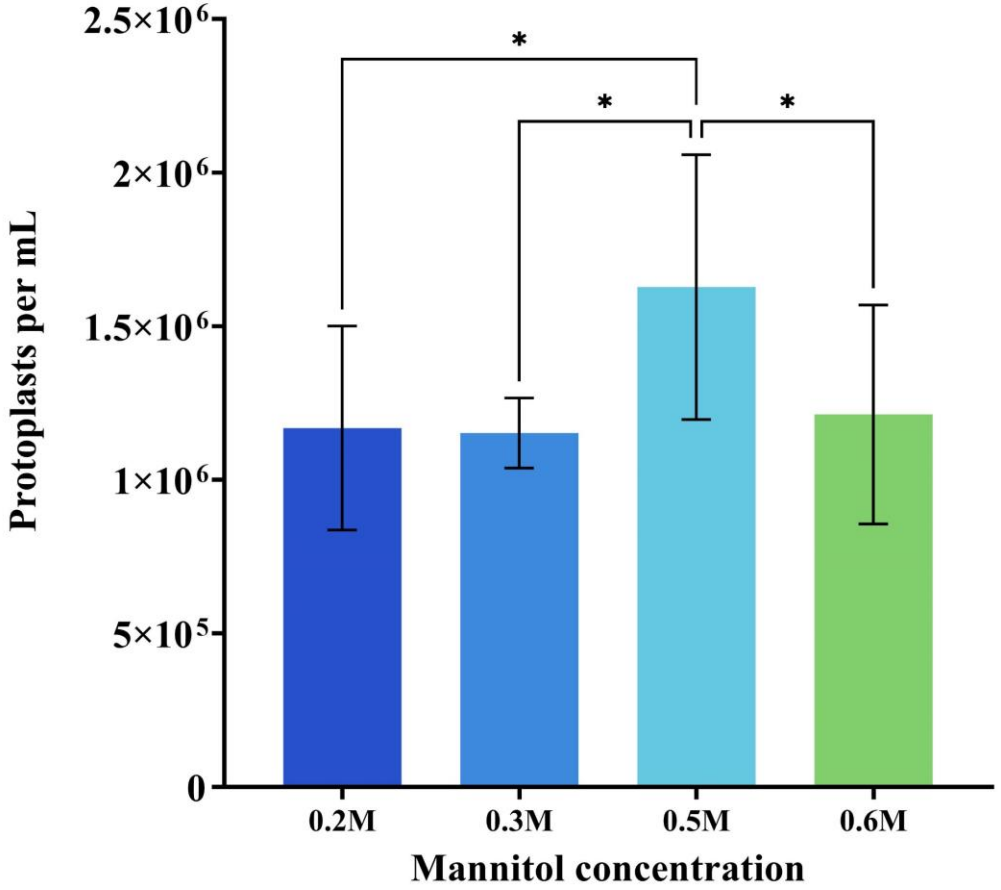
CELLULASE AND ENZYMATIC TREATMENT



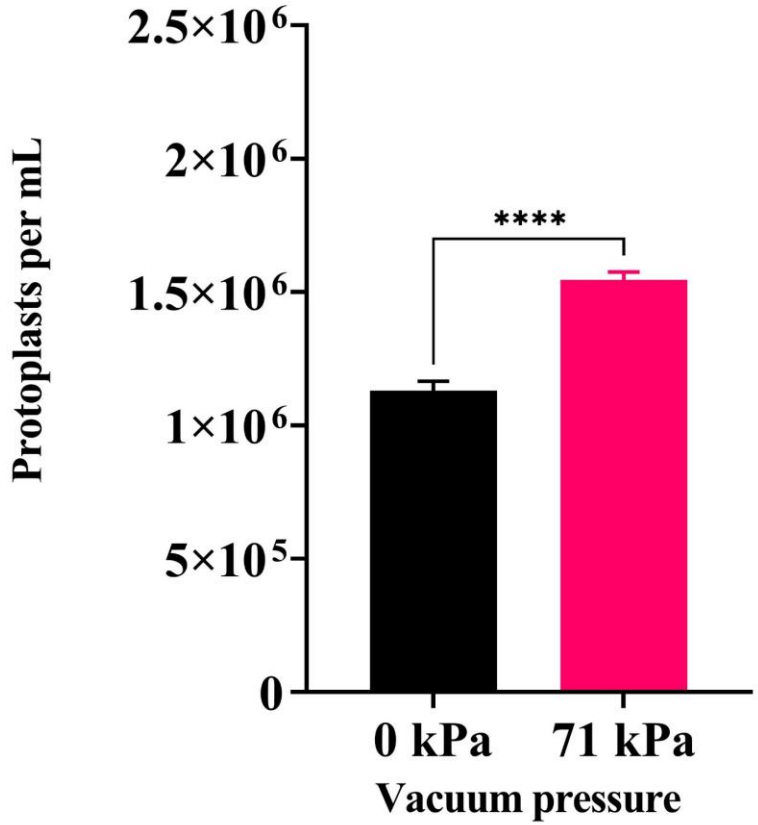


MANNITOL AND VACUUM INFILTRATION

a) Viable protoplasts after mannitol pretreatment

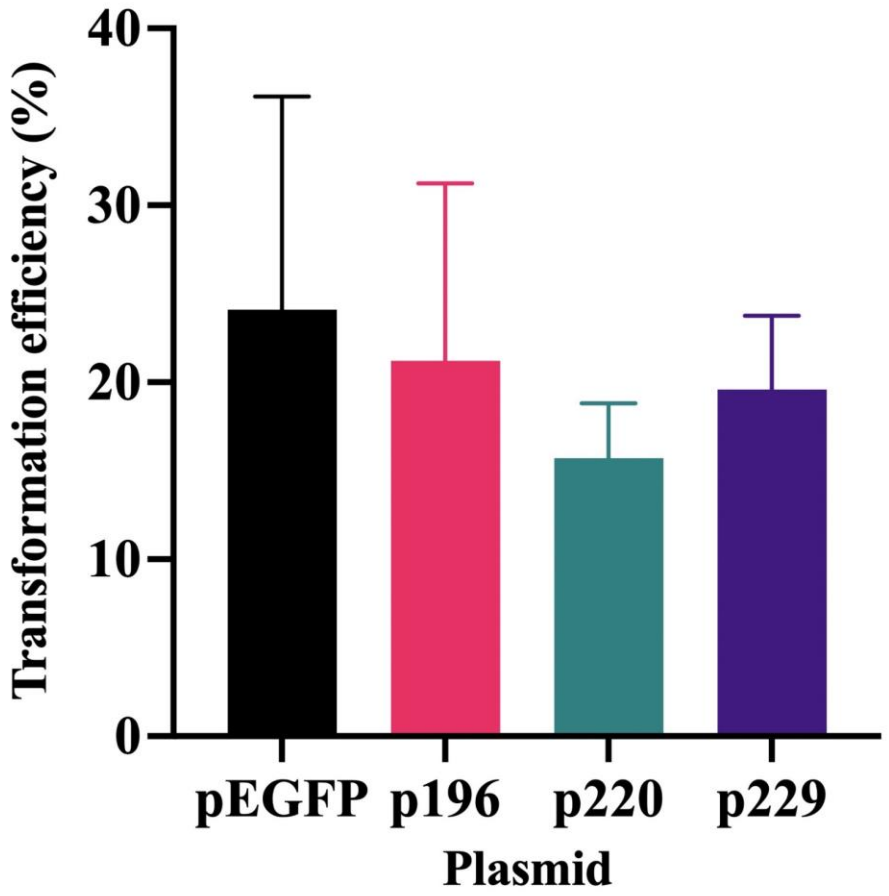


b) Viable protoplasts after vacuum infiltration

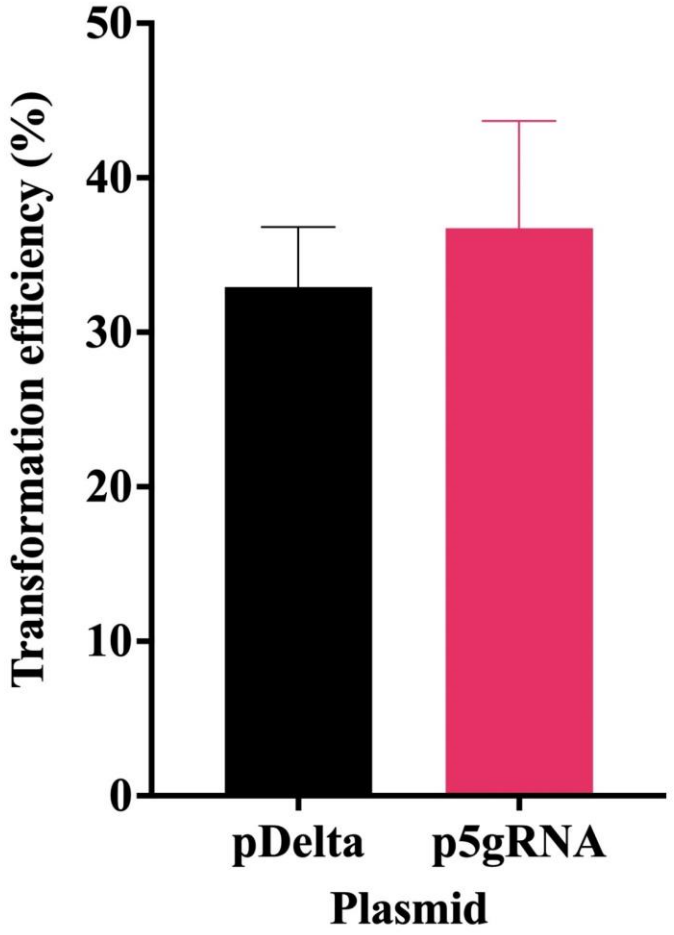


PEG-MEDIATED TRANSFORMATION

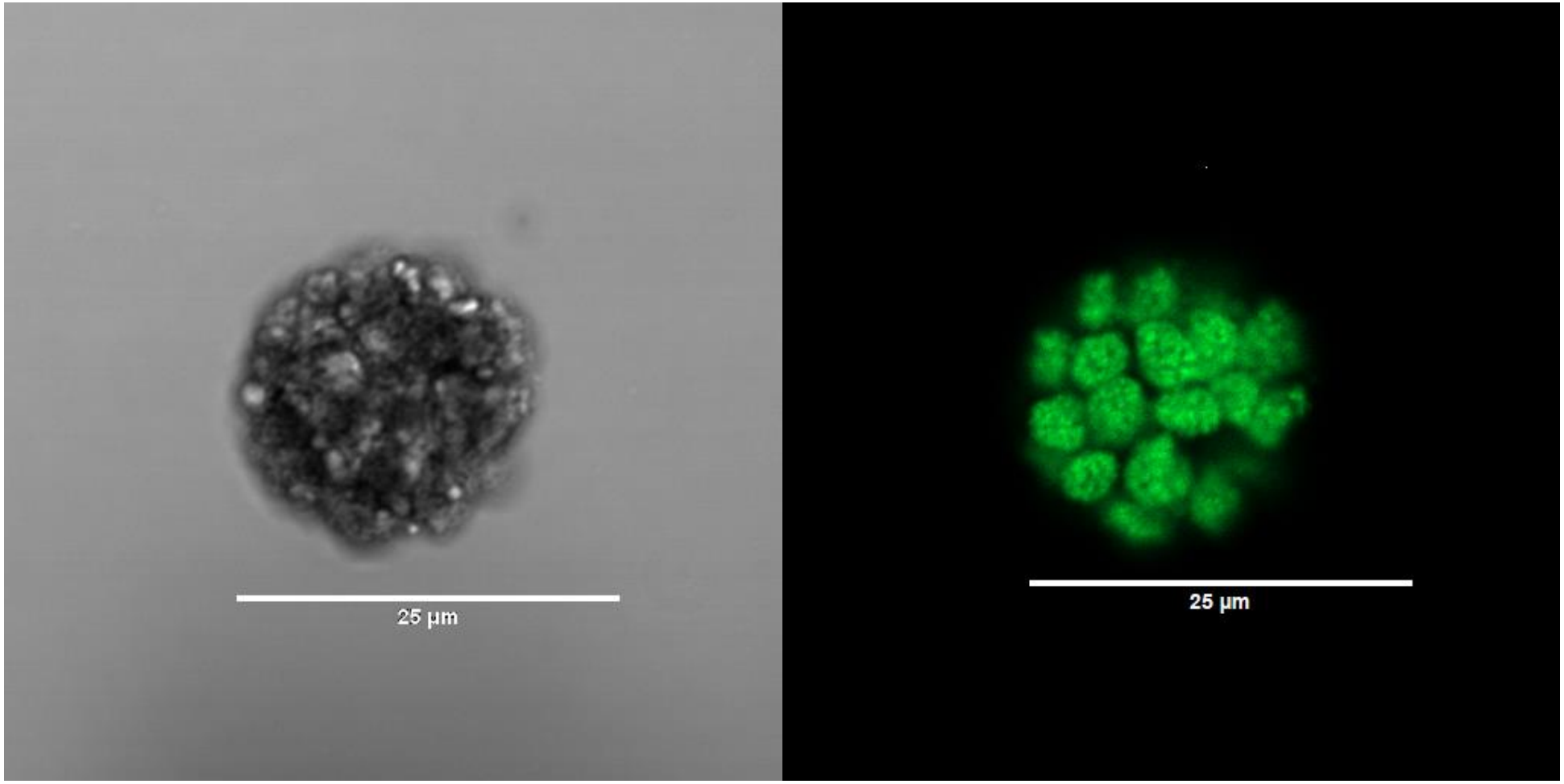
a) Transformation efficiency of pHSE401/EGFP



b) Transformation efficiency of the ZsGreen plasmid



ZsGREEN FLUORESCENCE



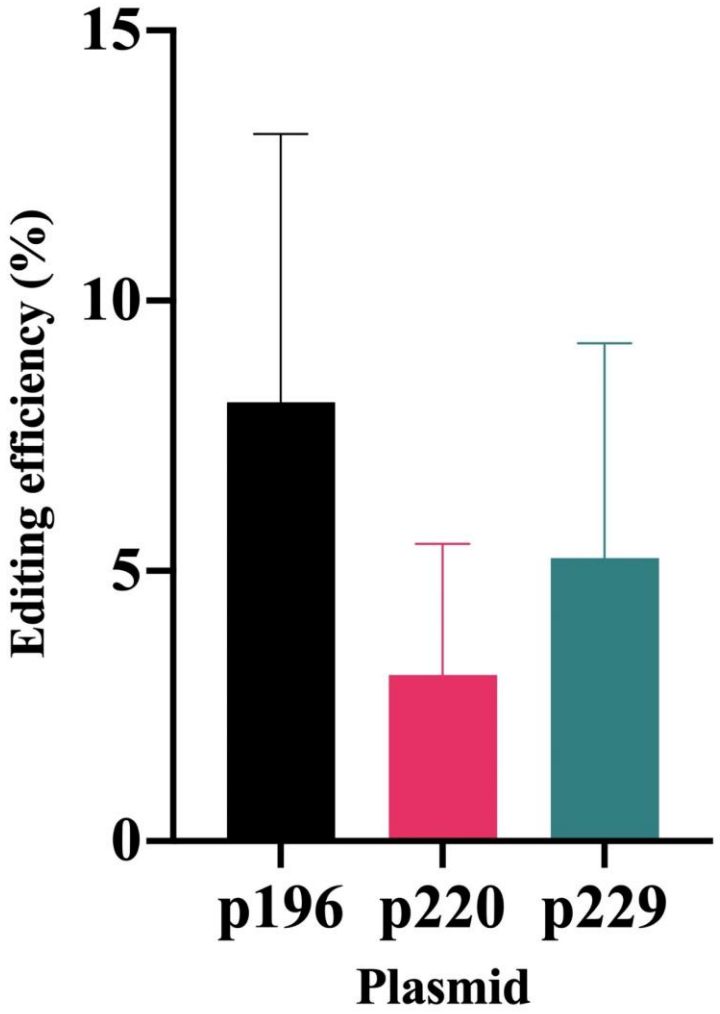
Brightfield

Fluorescence

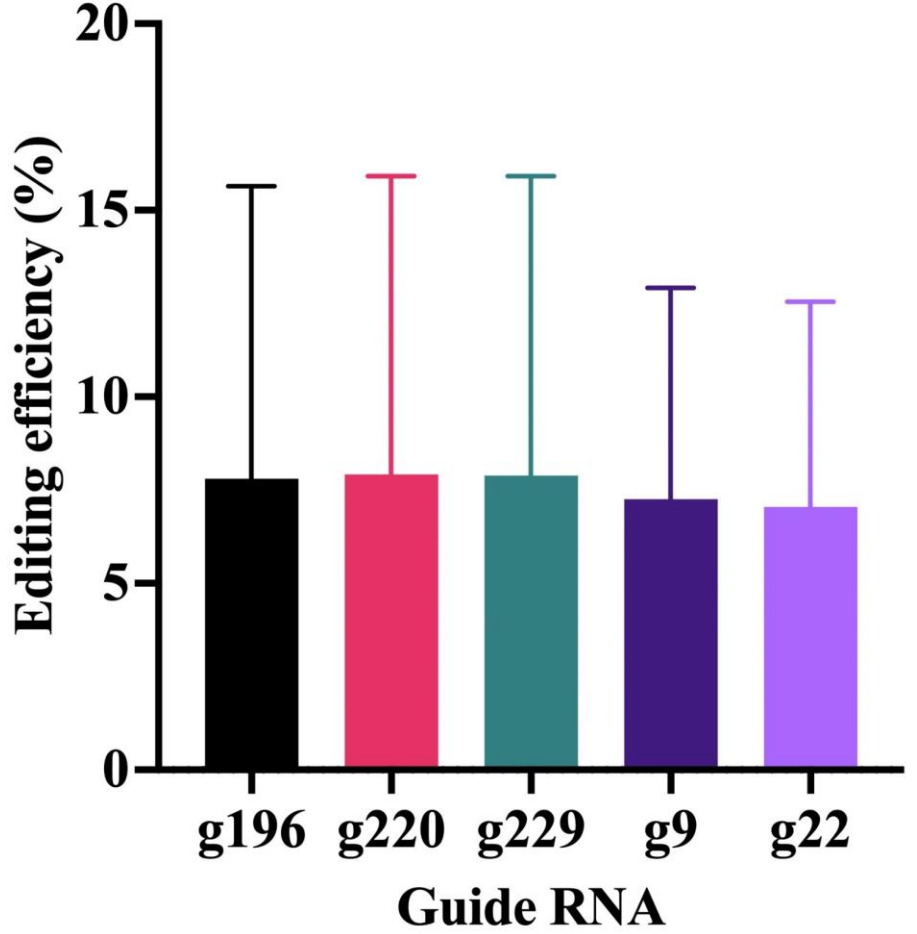
TAL TECH

EFFICIENCY OF THE DIFFERENT VECTORS AND gRNAs

a) Editing efficiency of pHSE401/EGFP



b) Editing efficiency of p5gRNA



FINAL REMARKS

- Successful PEG-mediated transformation
  - More than 20% of the treated cells were transformed (had fluorescence)
- The DNA directly extracted from the transformed cells
  - Suitable for amplicon sequencing
  - Good quality for TIDE analysis
- Editing efficiency was calculated with TIDE for all the gRNAs
  - On average, around 7% of the sequences presented indels

TAL TECH

The proposed method can be used to evaluate the performance of guide RNAs in vivo with the aim of selecting the most suitable material for later transformation experiments.

TAL TECH

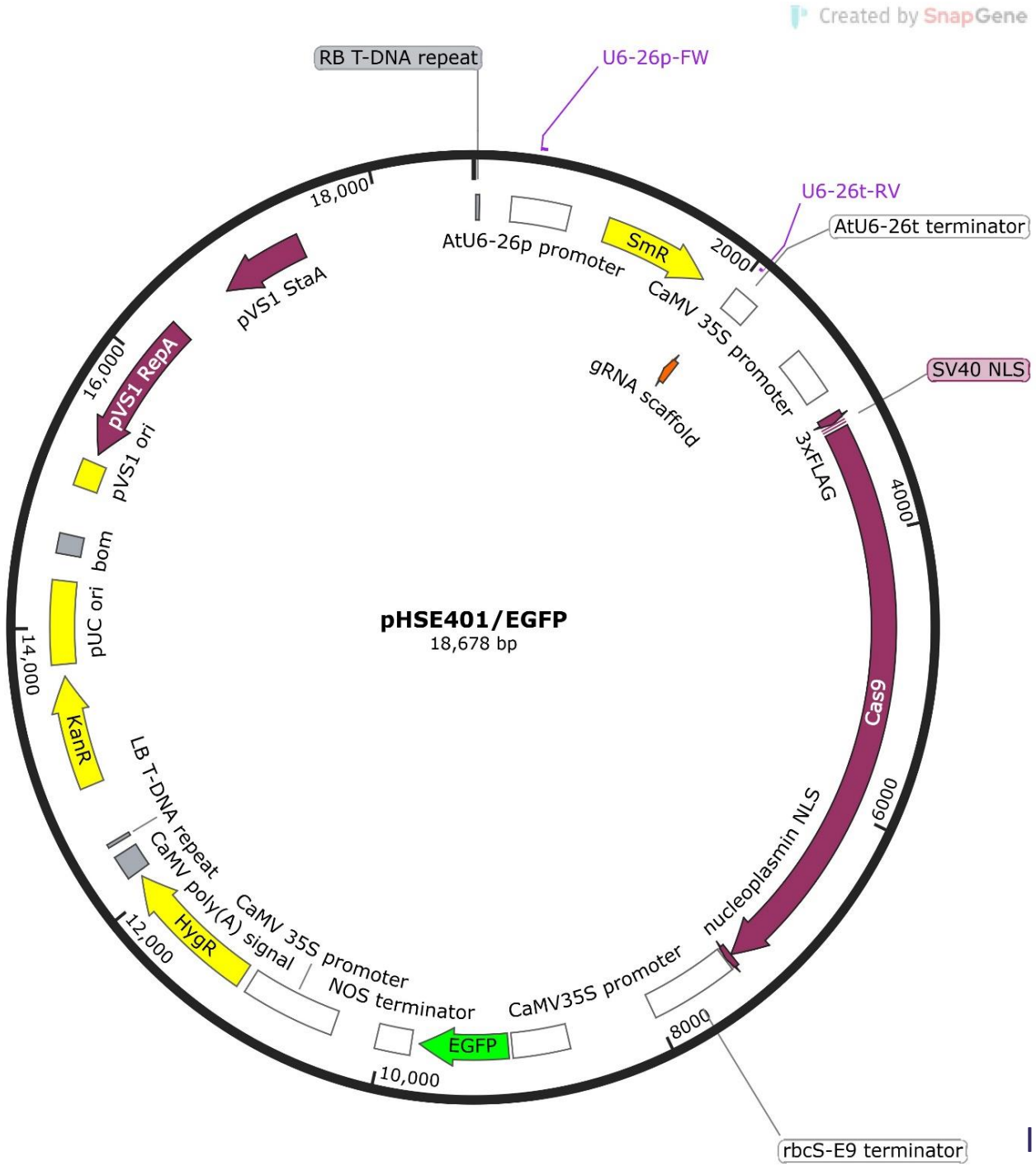
Iceland Liechtenstein Norway grants

Eesti Teadusagentuur
Estonian Research Council

EDIT GRASS 4FOOD

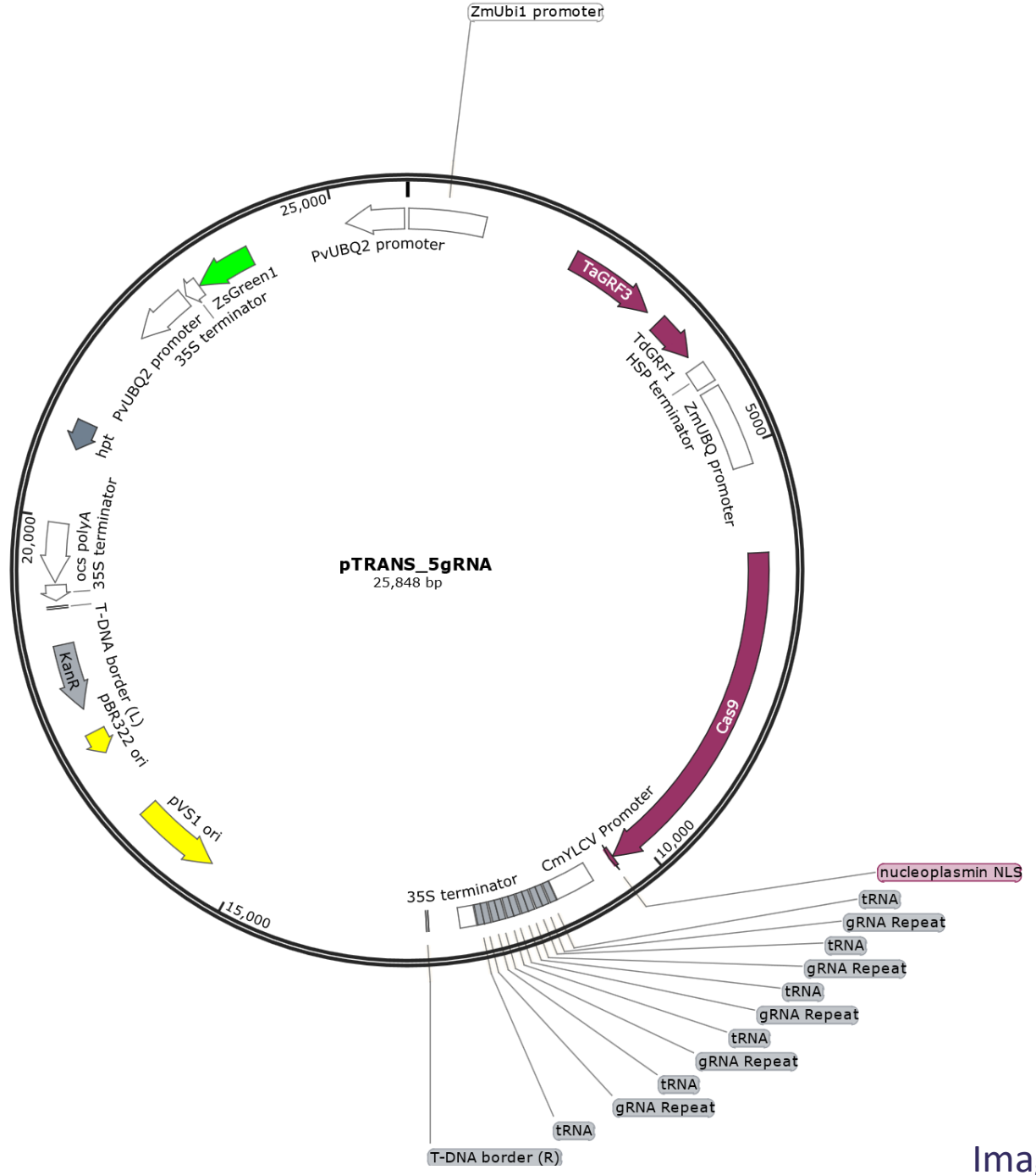
- Cecilia Sarmiento
- Erki Eelmets
- Anete Borodušķe
- Sergei Kushnir
- Nils Rostoks
- Lenne Nigul
- Signe Nöu

TAL TECH



TAL TECH

Image generated with SnapGene



TAL TECH

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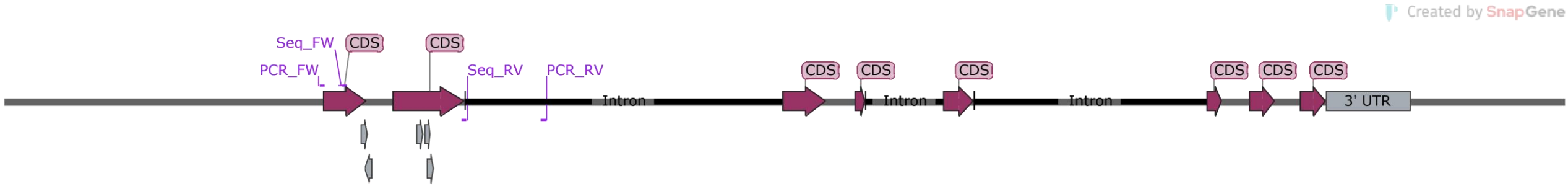

LpCBP20

TAL TECH

Image generated with SnapGene