Perennial ryegrass *in vitro* culture and protoplasts

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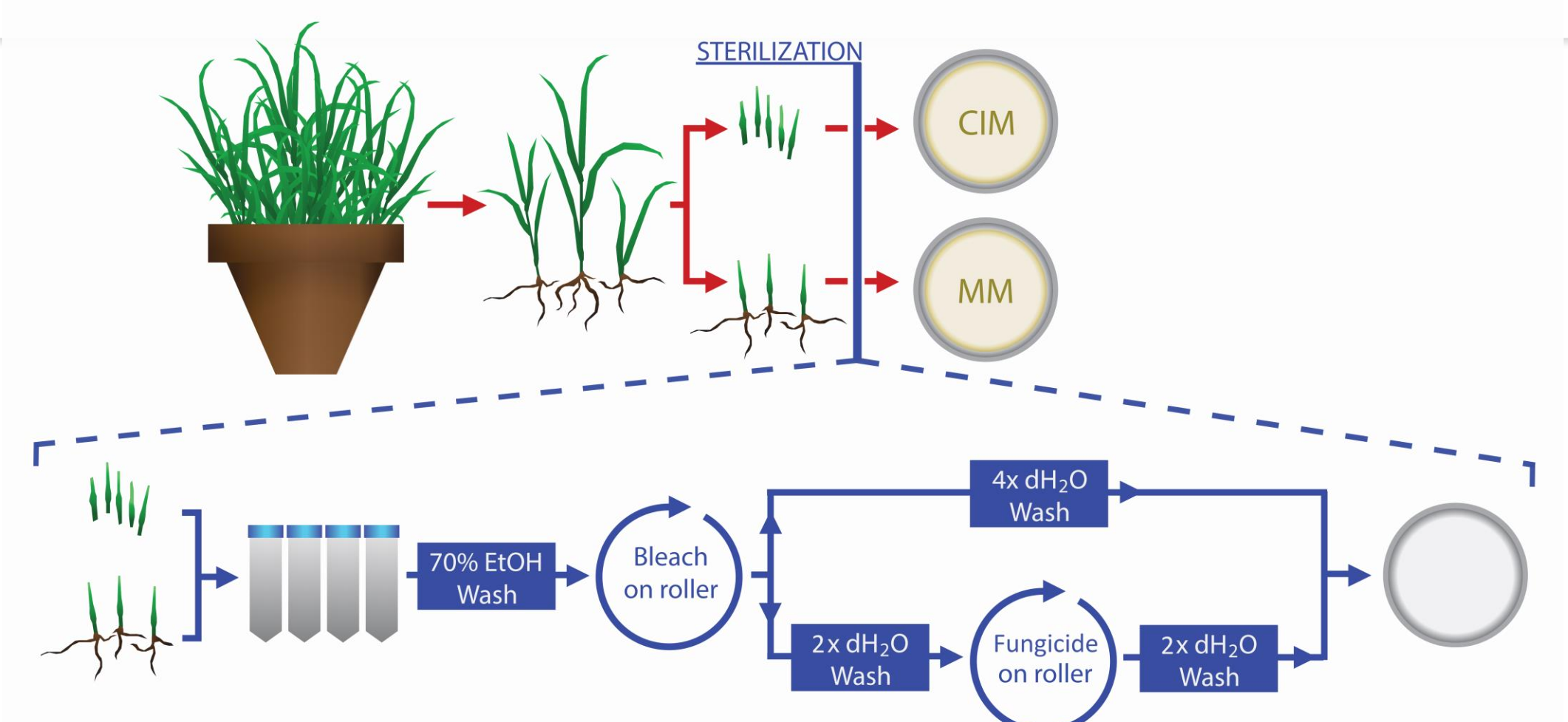
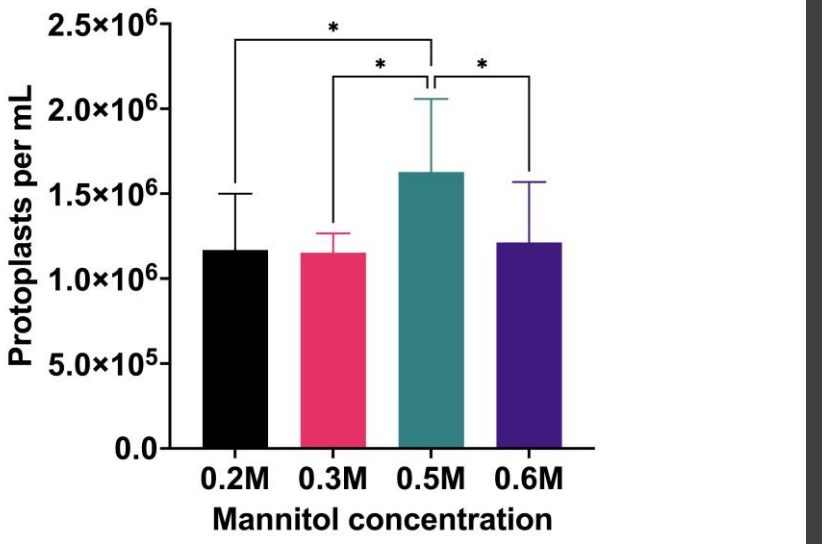
Functional characterization of frost and drought candidate genes in perennial ryegrass by CRISPR-Cas9

- A protocol to regenerate, in an asexual manner, *L. perenne* that can be used for gene editing was established. *In vitro* culture of tillers and generation of calli from the shoot apical meristematic region of the tillers was set up.

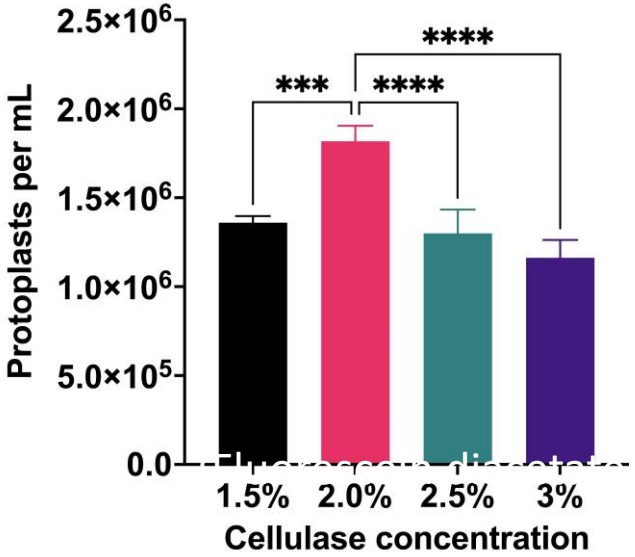


- Protoplasts are obtained from tillers.
- Protoplasts are used for the evaluation of the efficiency of different gRNAs. PEG transformation.
- **Need to optimize the protocol for the isolation of protoplasts.**

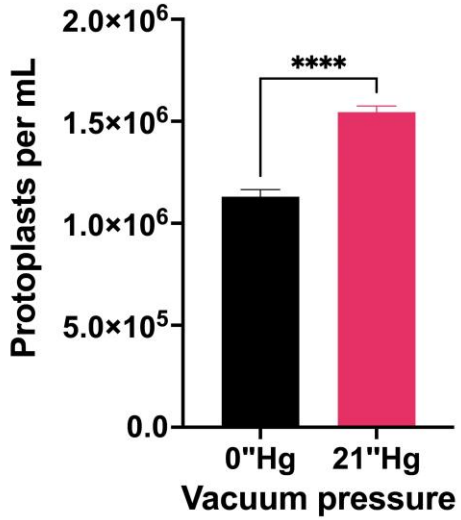
Viable protoplasts using different mannitol pretreatments

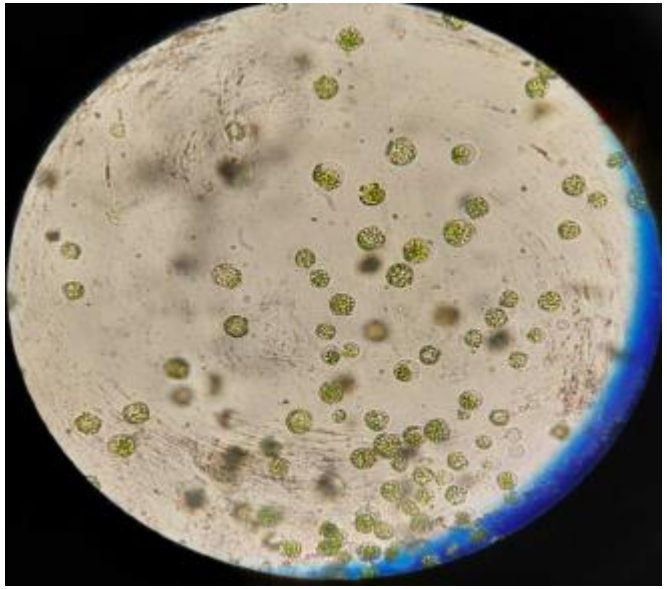


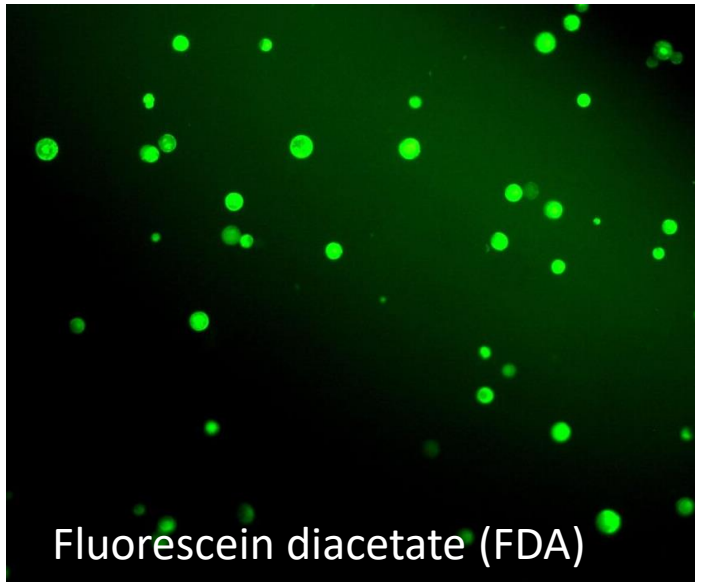
Viable protoplasts using different cellulase concentrations (8h treatment)

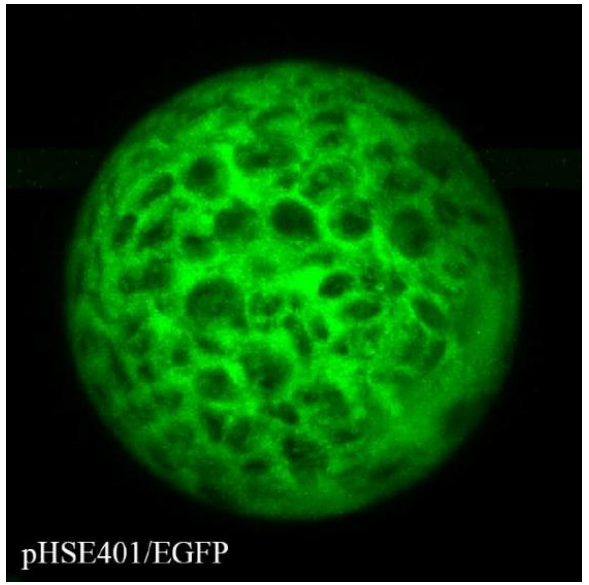


Viable protoplasts after vacuum treatment





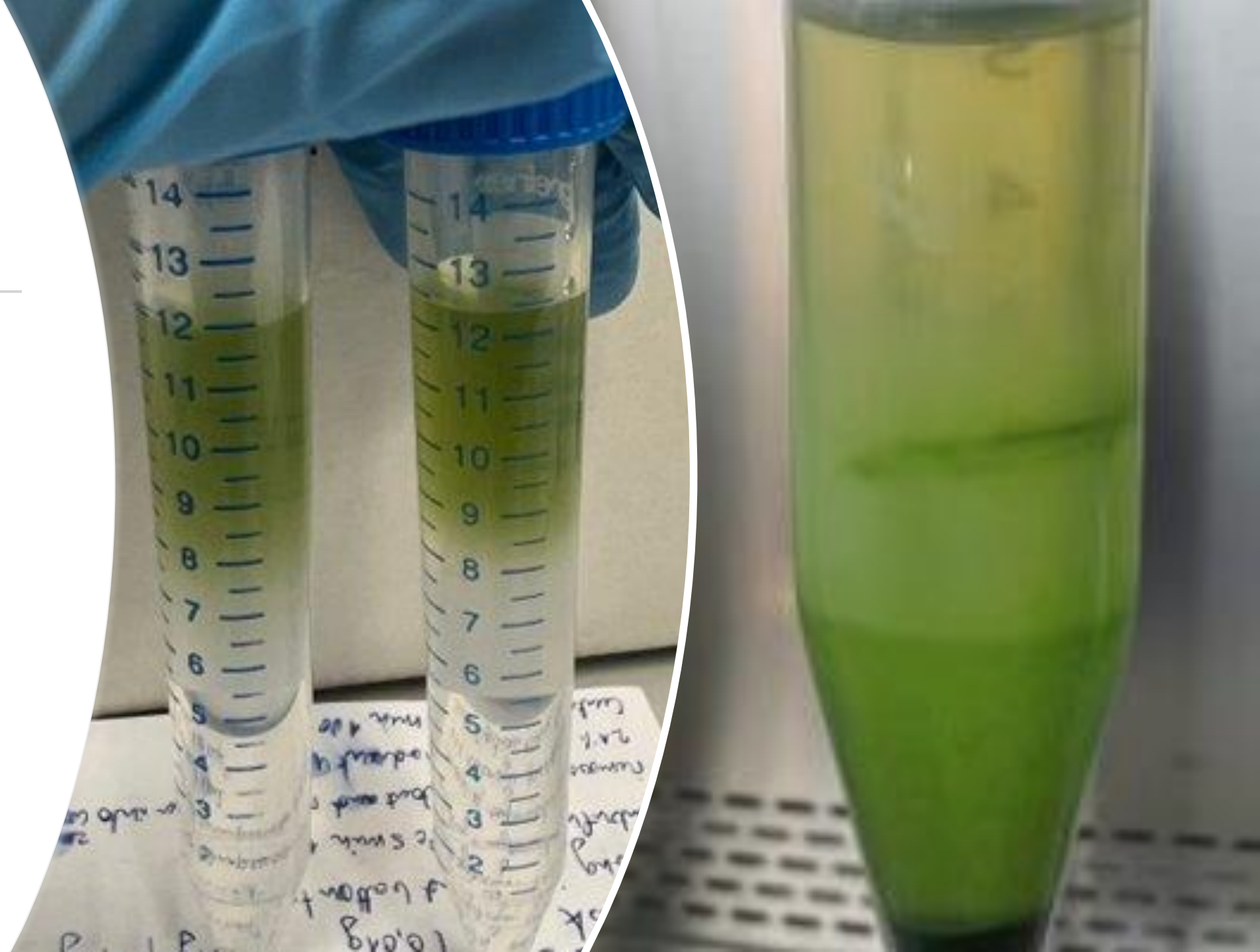




Reduction of debris in the protoplasts' suspension:

- 100 µm nylon filter
- Layering protoplasts suspension on top of 21% sucrose solution
- Centrifugation 100g for 10 minutes at 11ºC

("sucrose cushion")



PROTOPLASTS TRANSFORMATION

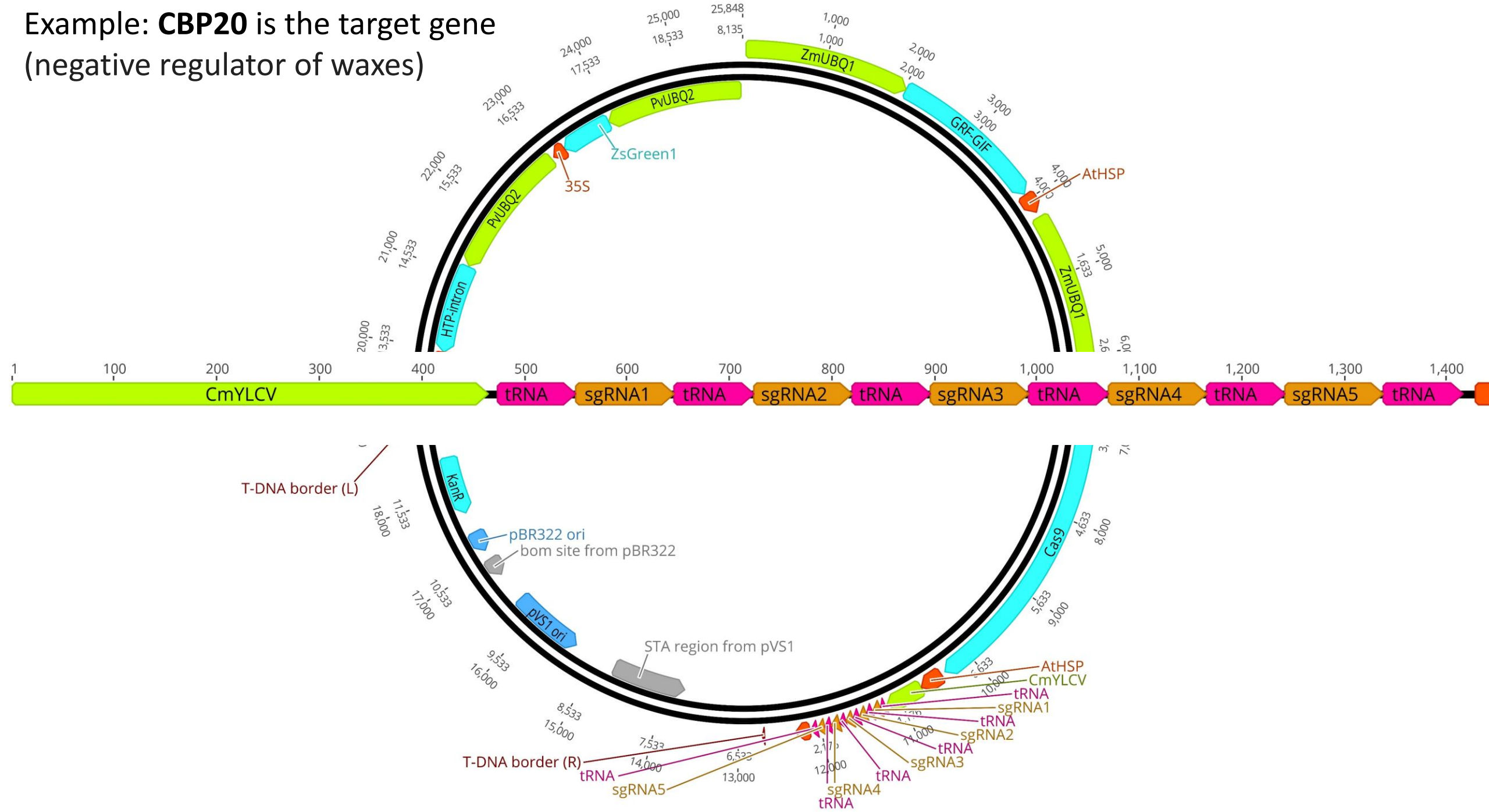
- PEG 4000 40% (w/v)
- D-mannitol 0.2M
- CaCl2 0.1M

METHOD

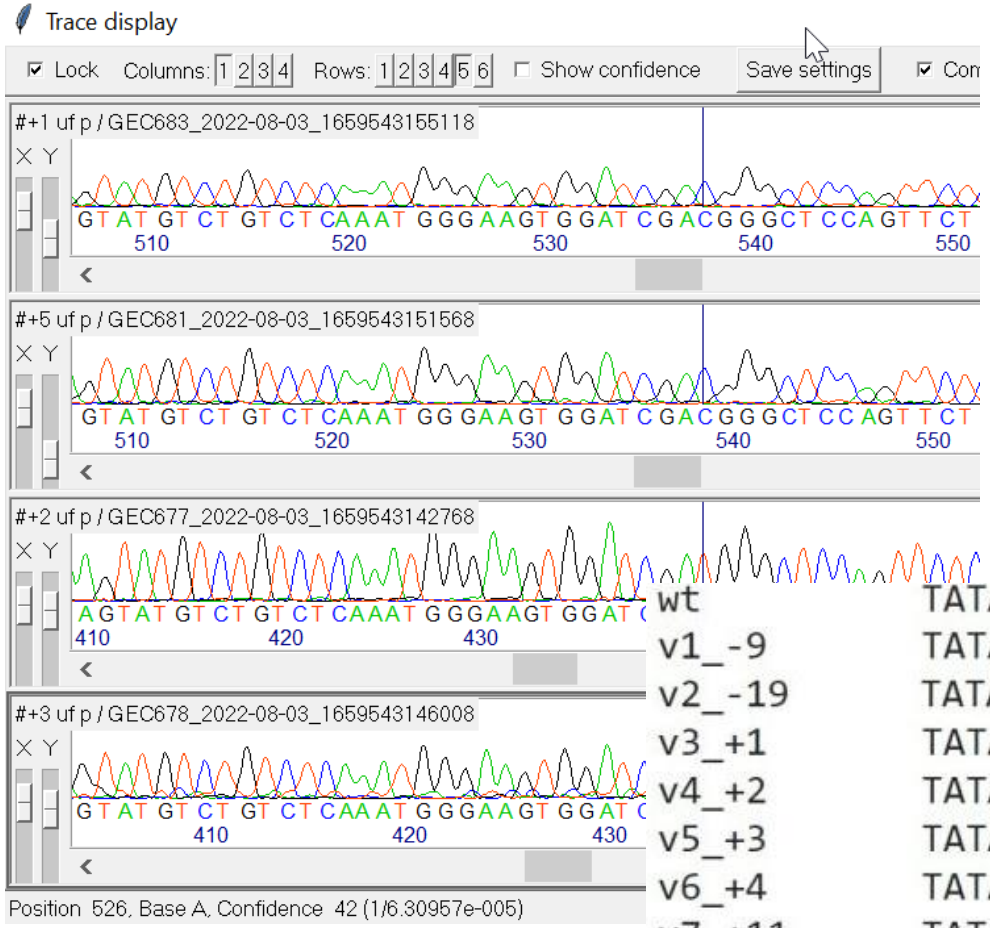
PLASMIDS

- Factors to improve the regeneration efficiency of calli (e.g. GRF4-GIF1).
- Reporters (e.g. GFP).
- Different numbers of gRNAs to target one gene (1-5).

Example: **CBP20** is the target gene
(negative regulator of waxes)



- Usually, the last annotated perennial ryegrass genomes are used as reference genome.
- Design (CRISPOR): First exon is targeted.
- Amplicon sequencing (Sanger for now). Benchling, ICE and TIDE are used for the analysis.

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wt     TATAGCCGACGAAGTACGTAGTCAC                ACTCACTAGTCACTGCAC  43
v1_-9  TATAGCCGACGAAGTACGTAGTCAC                --------TCACTGCAC  34
v2_-19 TATAGCCGACGAAGTACGTAGTCAC----------------ACTCACTAGTCACTGCAC  24
v3_+1  TATAGCCGACGAAGTACGTAGTCACN                ACTCACTAGTCACTGCAC  44
v4_+2  TATAGCCGACGAAGTACGTAGTCACNN               ACTCACTAGTCACTGCAC  45
v5_+3  TATAGCCGACGAAGTACGTAGTCACNNN              ACTCACTAGTCACTGCAC  46
v6_+4  TATAGCCGACGAAGTACGTAGTCACNNNN             ACTCACTAGTCACTGCAC  47
v7_+11 TATAGCCGACGAAGTACGTAGTCACNNNNNNNNNNN      ACTCACTAGTCACTGCAC  54
v8_+17 TATAGCCGACGAAGTACGTAGTCACNNNNNNNNNNNNNNNN ACTCACTAGTCACTGCAC  60
       ********************** ******************
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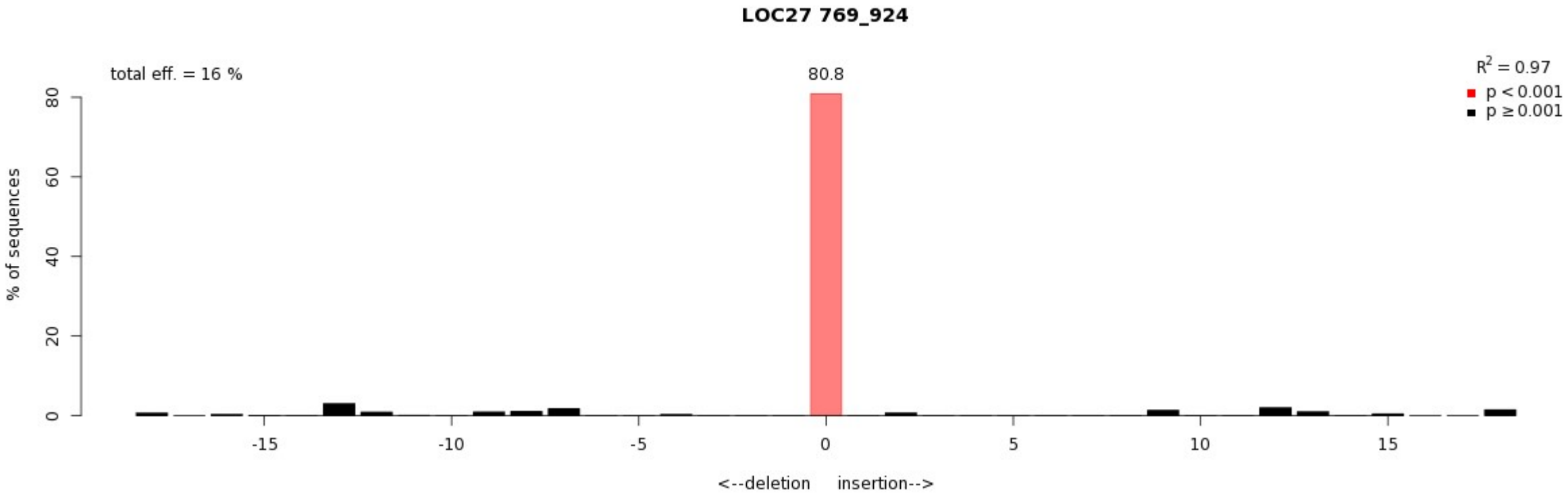
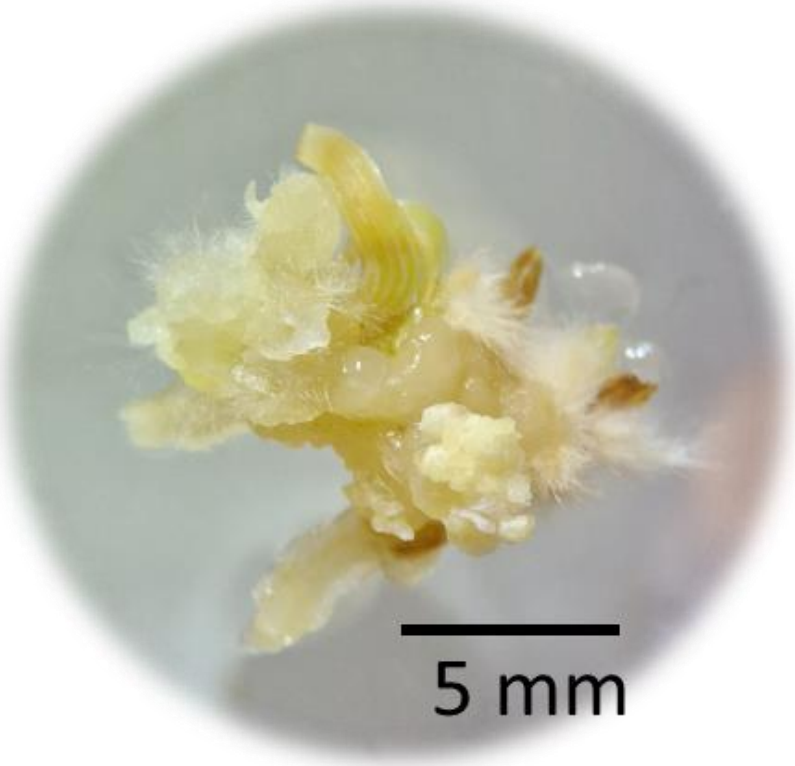
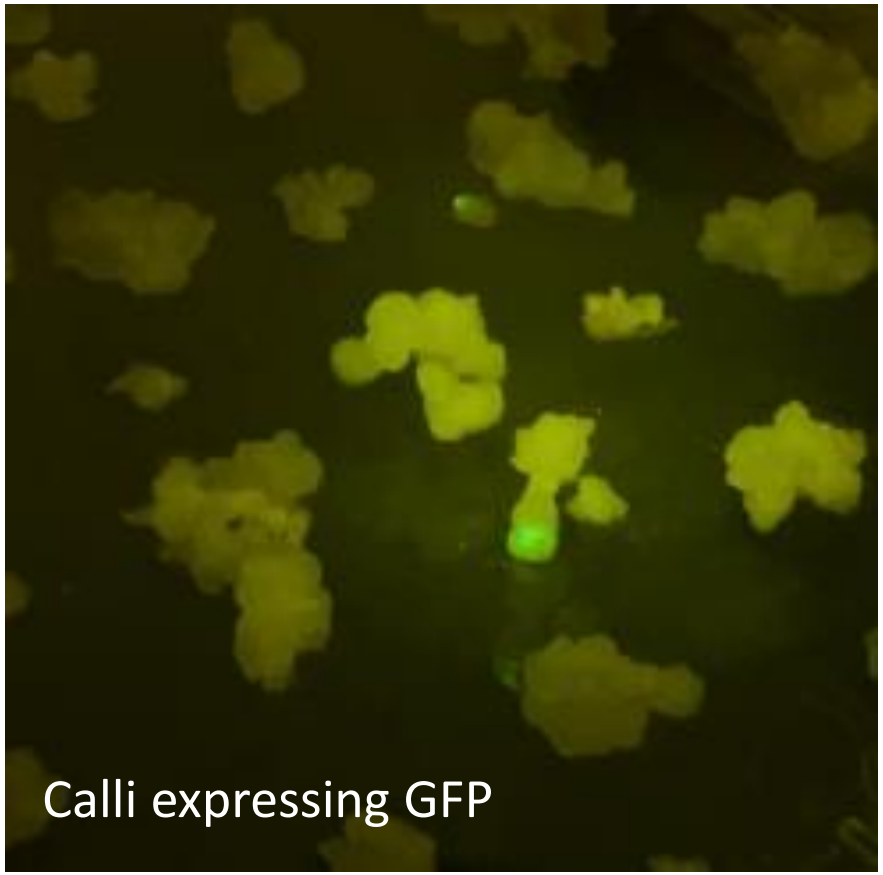
Indel Spectrum



The efficiency of editing in protoplasts is around 15%.
Next step: calli transformation.





Calli obtained from the SAM of tillers

Basic T-DNA design

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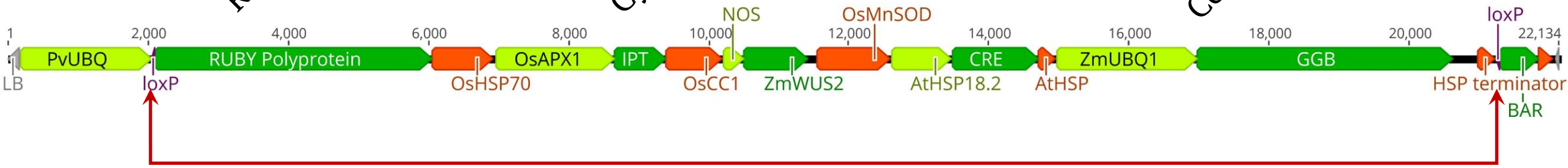
Red color visual marker
RUBY, Betalain biosynthesis

Cytokinin biosynthesis
IPT, Isopentyl Transferase

Stem cell fate
WUS2, WUSCHEL-like

Recombination at *loxP* sites
CRE, P1 phage recombinase

Cell proliferation & Embryogenesis
GGB, GRF-GIF-BBM polyprotein



Expected deletion at *loxP* sites after heat-shock treatment

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Differences in growth rates

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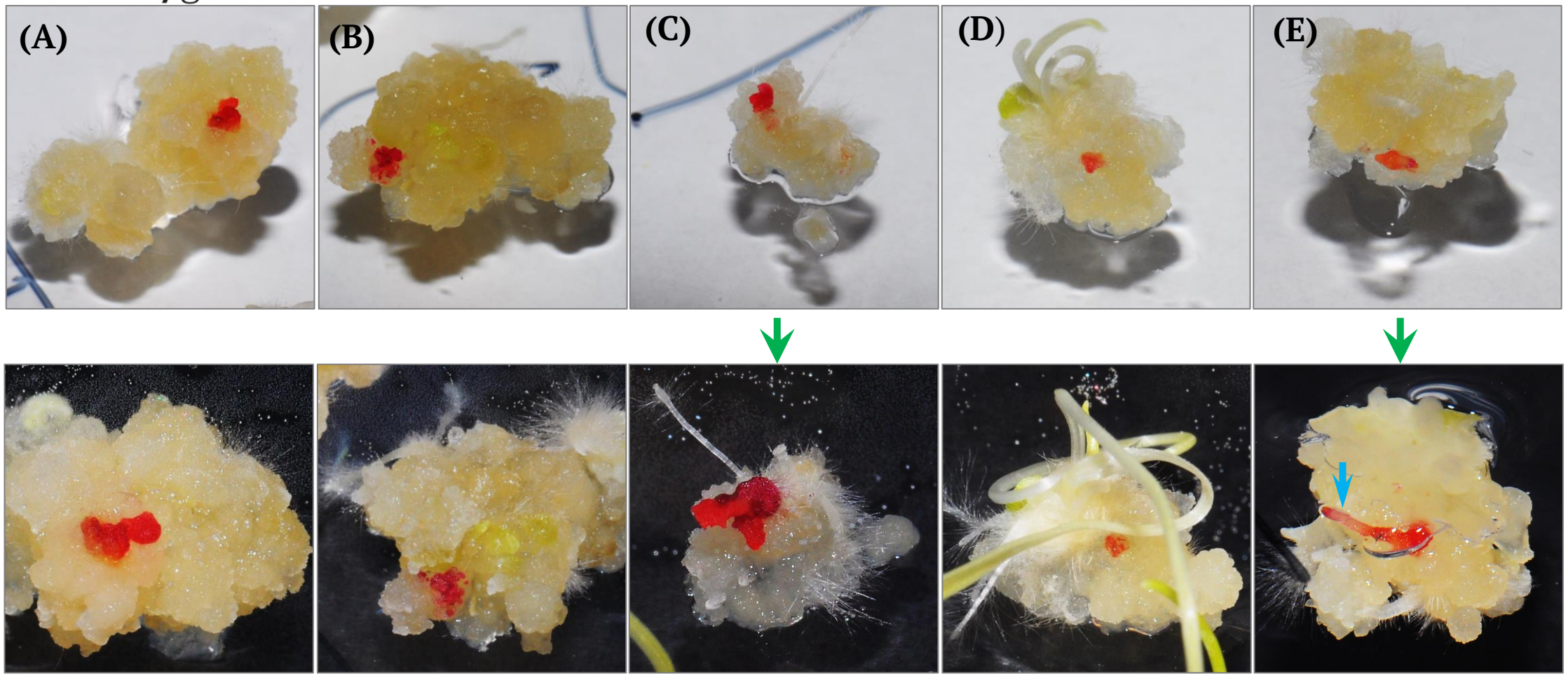

Ryegrass cells of the SSDX3 clone were mixed with *Agrobacterium tumefaciens* EHA105(pL2WOX4C1) on September 21st

Growing and differentiating clones. The clone in (E) formed a root appearing as a germinating embryo ( ).

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On hormone-free medium in one month

(Dr. Sergei Kushnir)

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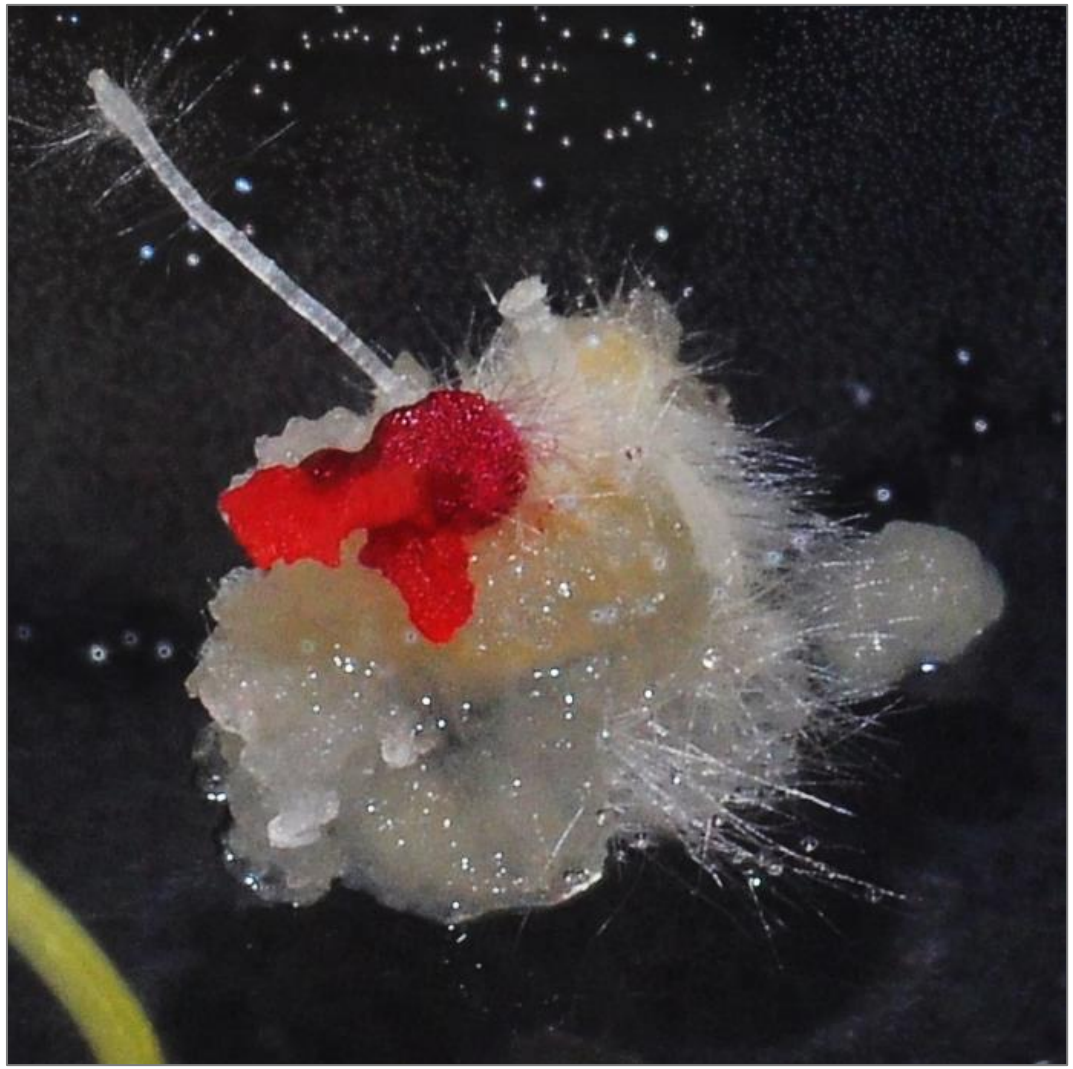

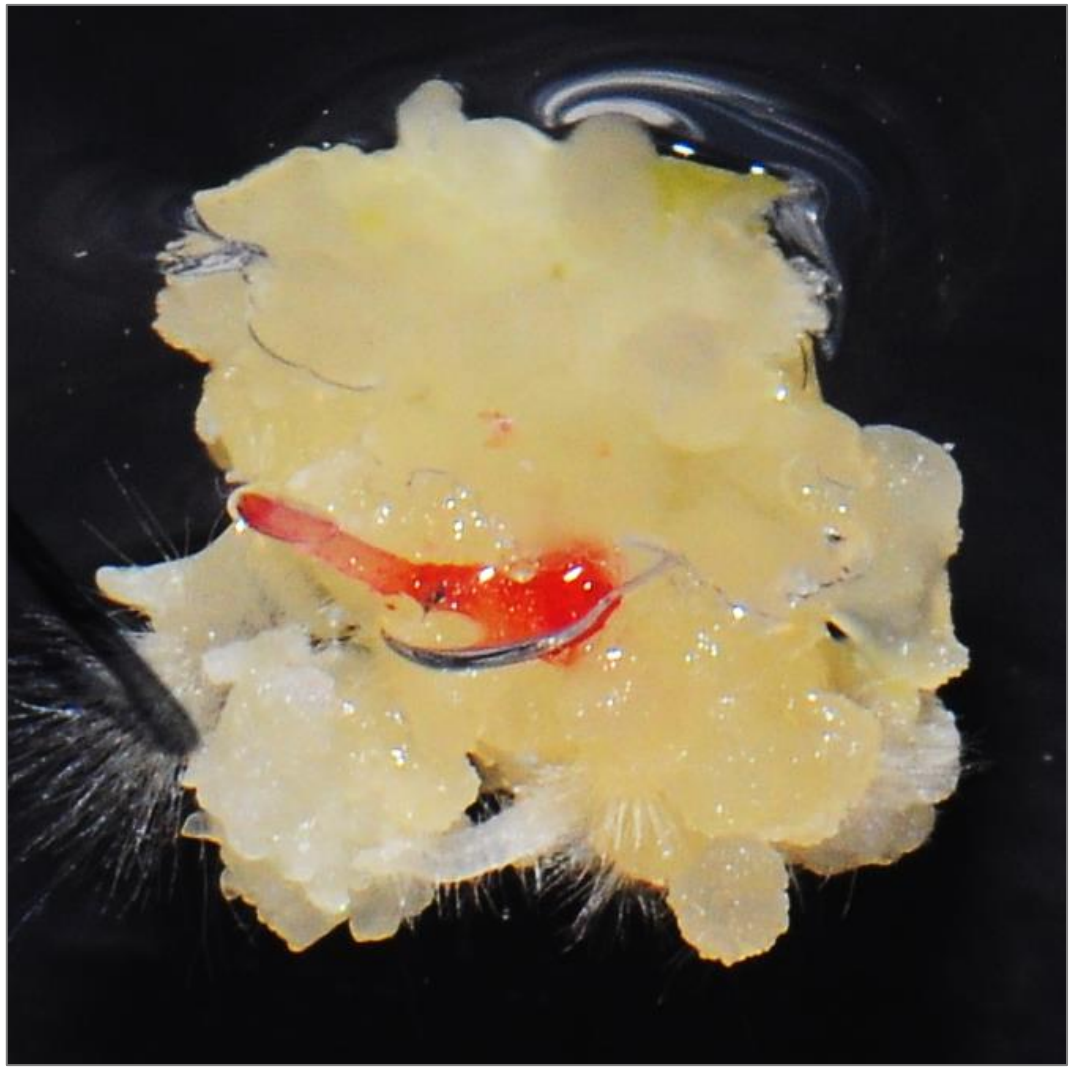

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