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Izglītības un zinātnes ministrija

Latvian Council of Science

Implementation of 8 projects funded by the EEA Baltic Research Programme in Latvia

18 October, 2023

EEA-RESEARCH-64

Improving adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR-Cas9 technology (EditGrass4Food)

Improving adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR-Cas9 technology (EditGrass4Food)

EEA-RESEARCH-64

Principal Investigator: Nils Rostoks

Promoter: University of Latvia
Partners:

- Norwegian University of Life Sciences, NMBU, Norway
- Tallinn University of Technology, TalTech, Estonia
- Lithuanian Research Centre for Agriculture and Forestry, LAMMC, Lithuania

From **01.05.2021** to **30.04.2024** (36 months)

Website: https://www.editgrass4food.lu.lv/en/

Project goals and current progress

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Aim of the project is to utilize transcriptomics and functional genomics to increase sustainability in agriculture through improvement of perennial ryegrass with better adaptation to frost and drought for current and future climates.

1. Establish a diverse perennial ryegrass core association panel by utilization of data from ongoing projects (WP1),
2. Screen the association panel in order to detect haplotype-resolved single-nucleotide variants and structural variation in the targeted genes/alleles for freezing and drought genes (WP1),
3. Identify novel genes and characterize drought and freezing tolerance genes by comparing their expression for pathway related genes in non-edited and mutant plants (WP2),
4. Develop CRISPR-Cas9 constructs and generate CRISPR-edited perennial ryegrass mutants for freezing and mild drought tolerance (WP3),
5. Validate and characterize the role of the genes and their sequence variations in the freezing and drought mechanisms (WP4).

Work packages

WP1. Establishment and screening of perennial ryegrass association panel for freezing and drought related traits. Coordinator: NMBU; Involved partners: NMBU, LAMMC

WP2. Transcriptome regulation of freezing and drought tolerance in perennial ryegrass. Coordinator: NMBU; Involved partners: NMBU, LAMMC

WP3. Functional characterization of frost and drought candidate genes in perennial ryegrass by CRISPR-Cas9. Coordinator: TalTech; Involved partners: LU, NMBU

WP4. Validation of improved freezing and water shortage tolerance. Coordinator: LAMMC; Involved partners: TalTech, NMBU, LU

WP5. Management and coordination of research activities and dissemination of results. Coordinator: LU; Involved partners: TalTech, NMBU, LAMMC

Current progress of project activities – WP1

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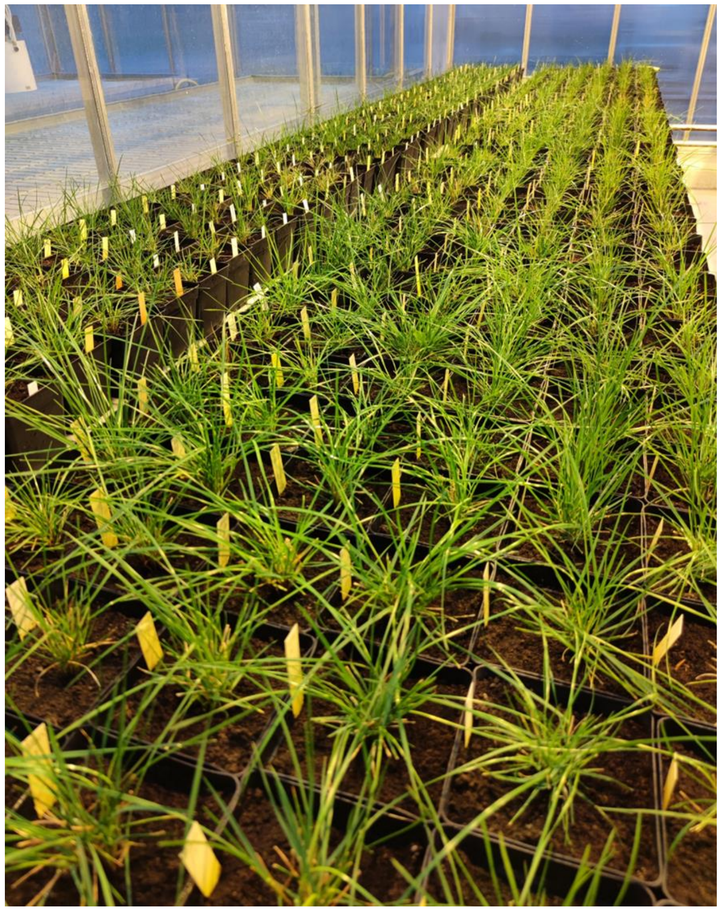
Establish a diverse perennial ryegrass core association panel by utilization of data from ongoing projects (Coordinator: NMBU; Involved partners: NMBU, LAMMC)

- NMBU and LAMMC have established an association panel of 325 individuals. The plant material was selected based on freezing and drought data from previous projects, including Nordic public – private partnership project (https://www.nordgen.org/en/our-work/nordic-public-private-partnership-ppp/ppp-projects/). The plants were potted and grown in the greenhouse and DNA extractions were performed from the fresh leaf materials
- 10 genes related to frost and drought were selected for characterizing phenotypically contrasting genotypes using target sequencing.
- Currently target sequencing and bioinformatic analysis of data is underway



WP1: Establishment and screening of perennial ryegrass association panel for freezing and drought related traits

- Plant material:

- 50 genotypes (both frost and drought)
- Frost: 25 synthetic populations from PPP project
- Drought: 25 from PPP

WP1: Establishment and screening of perennial ryegrass association panel for freezing and drought related traits

- Genes list:

- Drought: PhyB, MYB41, NAC038, MYB94, WSD11, TSO1, MYB4, HSL1. WRKY49, PRR5

- Frost: VRN1, QM, TPT, IRI, CBF6, Dhn1, CBP60, GIGANTEA, LEA14, PRR95

Twist target seq approach

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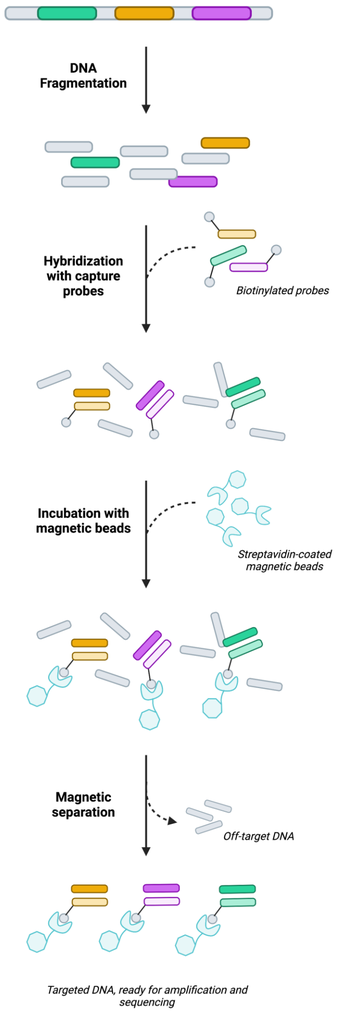
Hybrid Capture Target Enrichment Workflow



Panel design by Twist Bioscience

Target enrich your libraries using Twist long-read protocol[2]

DNA extraction and shearing → Library prep → Target enrichment

SMRTbell® library prep → HiFi sequencing

Construct and prepare SMRTbell library for HiFi sequencing[3]

PacBio tools available in SMRT® Link or command line[4]

- Demultiplex samples — lima
- Deduplicate HiFi reads — pbmarkdup
- Map to reference — pbmm2
- SV calling — pbsv

Industry standard command line tools

- Variant calling — DeepVariant[5]
- Haplotype phasing — WhatsHap[6]
- Target capture stats — HsMetrics Picard[7]

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Twist target seq approach

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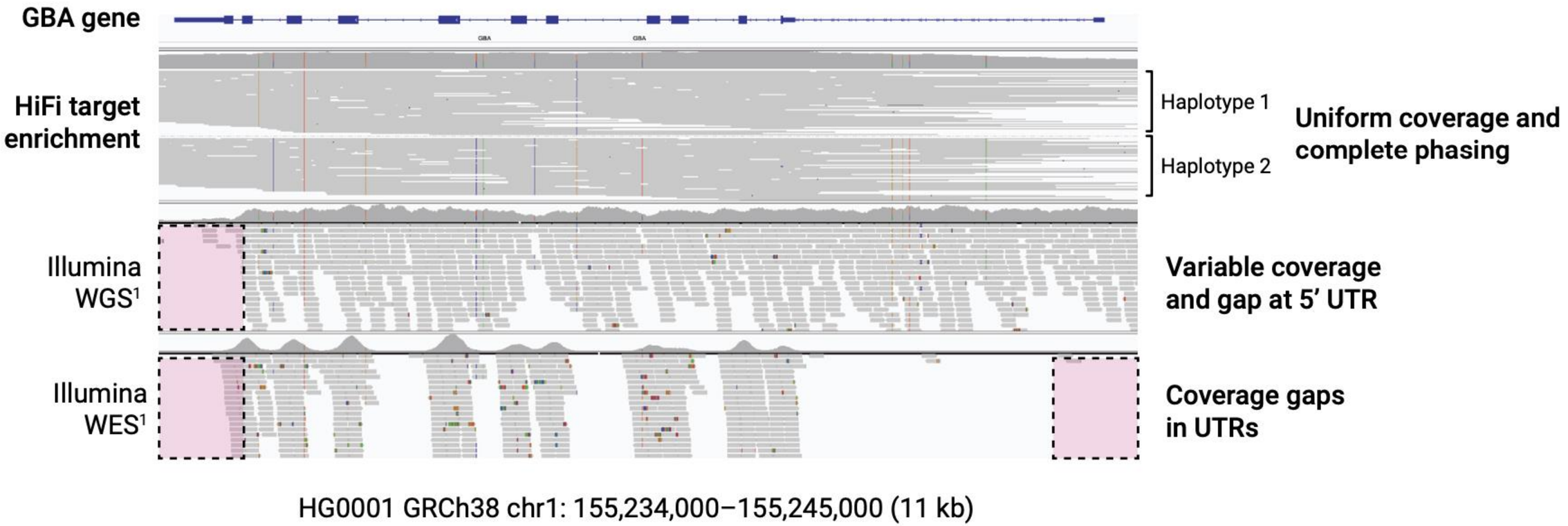

WP1: Progress so far.....

- Designed the gene panels for all the 10 genes.
- On average approximately 10kb size (including promoter, exon and intron regions)

- Tested twist target seq approach for 1 gene (CBF6) across 5 samples sequenced on PacBio HiFi seq
- Received the data to check the quality of the data
- Currently analzing the data for variations across these genotypes

- Once the results are satisfactory, will proceed with the full scale for all the genes across the samples.

Current progress of project activities – WP2

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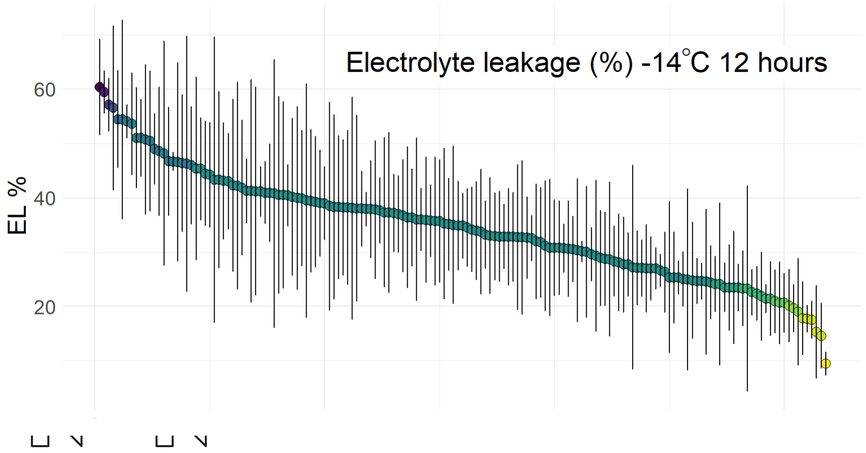
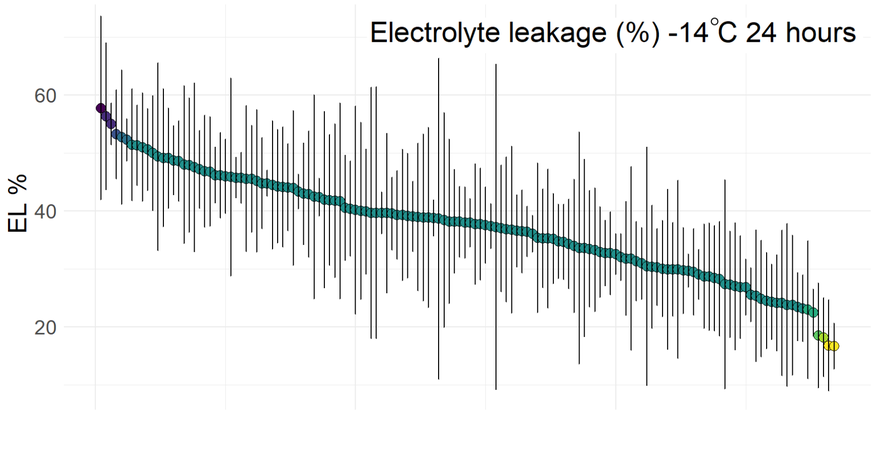
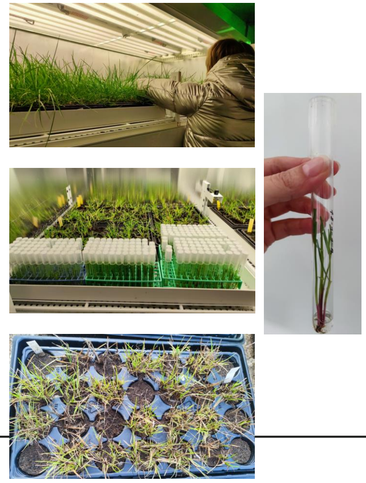
Transcriptome regulation of freezing and drought tolerance in perennial ryegrass (Coordinator: NMBU; Involved partners: NMBU, LAMMC)

- Phenotyping of the panel for freezing and drought related traits for associating with single nucleotide variants in targeted genes and to identify two most sensitive/resistant genotypes (completed)
  - freezing – electrolite leakage, survival rate
  - drought – leaf growth, stomata conductance, Fv/Fm (maximum quantum yield of photosynthetic system II)
- RNA extraction, sequencing library preparation and Illumina NGS under way.







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WP2: Transcriptome regulation of freezing and drought tolerance in perennial ryegrass

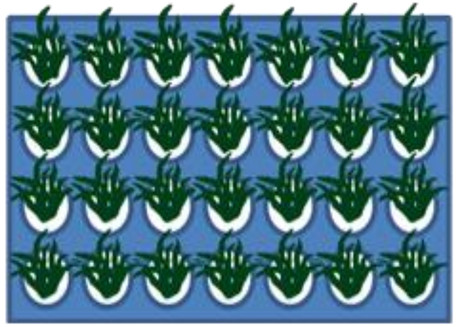
- Two freezing tolerant and two susceptible genotypes are selected and grown at short days (8 h) at 18°C to gain biomass. The plants are pre-acclimated for two weeks at 4°C before subjected to freezing at -8 or -12°C.
- Freezing tests are performed in growth chambers at LAMMC
- After 12 hours of stress, electrolyte leakage is measured and plants are transferred back to control conditions, grown for 3 weeks and scored for freeze damage from 0 (dead) to 9 (no damage). Samples of leaf tissue of each genotype are taken for RNA extractions at the day before stress onset and 8 hours after onset of low temperatures.
- RNAseq data is generated for 72 samples (including 3 replicates) and currently under analysis to identify differentially expressed genes between resistant and susceptible genotypes.
- Specifically focusing on gene expression before and after cold acclimation and also at -5 and -10 oC of frost.

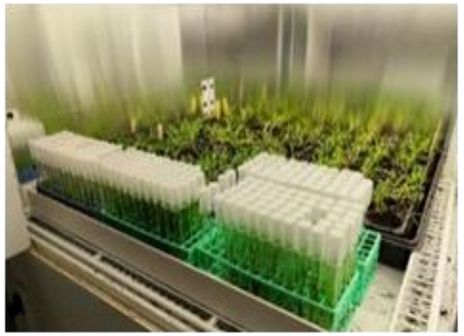
WP2: Transcriptome regulation of freezing and drought tolerance in perennial ryegrass.

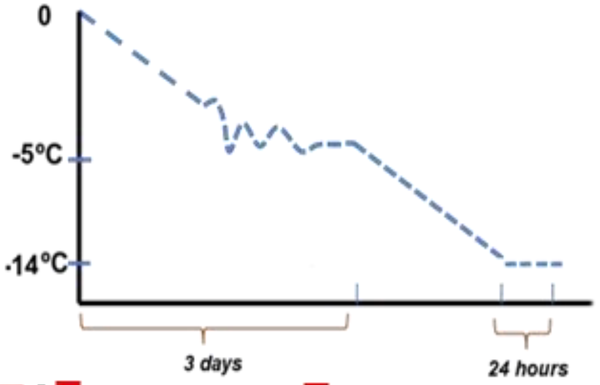
- Two drought tolerant and 2 susceptible genotypes are measured for leaf growth under drought conditions for 5 days and leaf samples for RNA extraction will be taken every day.
- RNA will be extracted from flash-frozen leaves. Strand-specific libraries will be created and sequenced across all lanes using Illumina paired-end sequencing.
- We will employ the pipeline developed by Kovi et al. (2017) for analysis of differential expression.

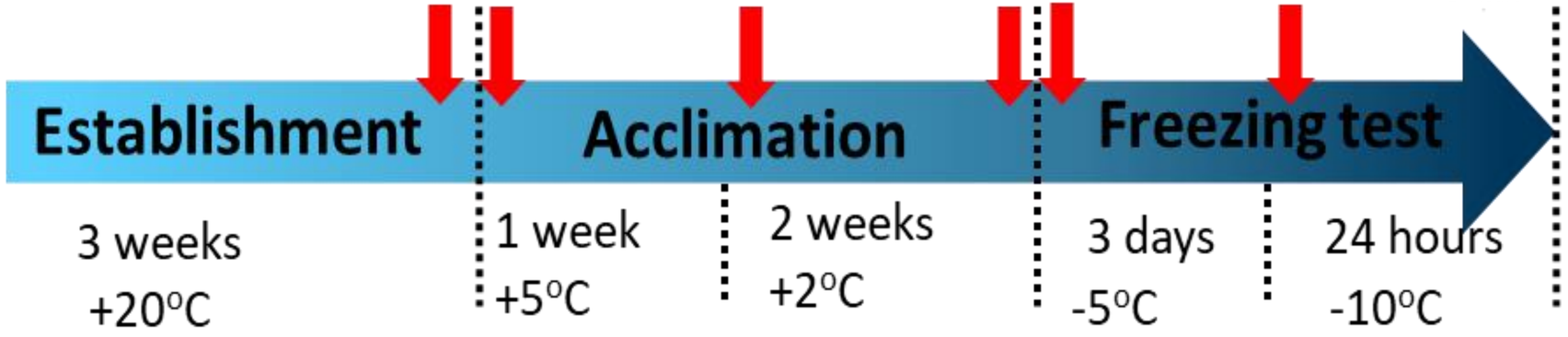
Sampling for freezing transcriptome analysis

4 genotypes, selected based on electrolyte leakage after freezing test





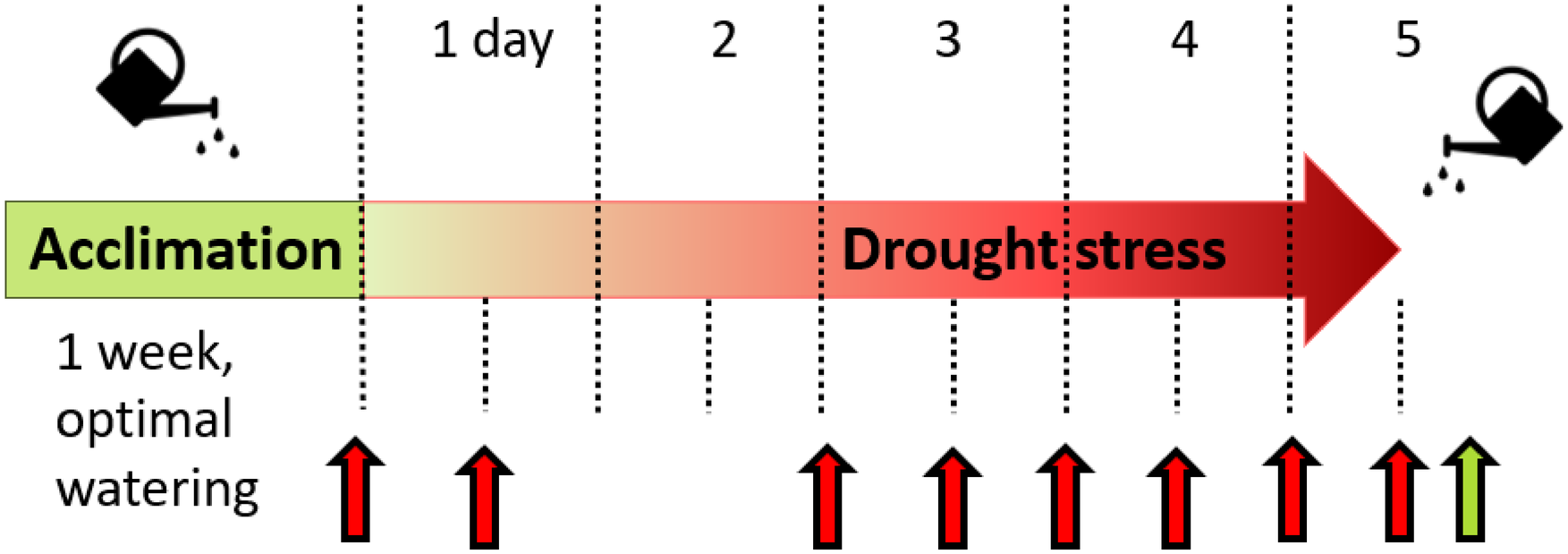




- sampling point

Sampling for drought transcriptome analysis

4 genotypes, selected based of leaf elongation rate under mild drought and relative water content, Fv:Fm under severe drought



Two samplings per day: morning and afternoon. Last sampling – regrowth 2 h after watering

Sampling point

Current progress of project activities – WP3

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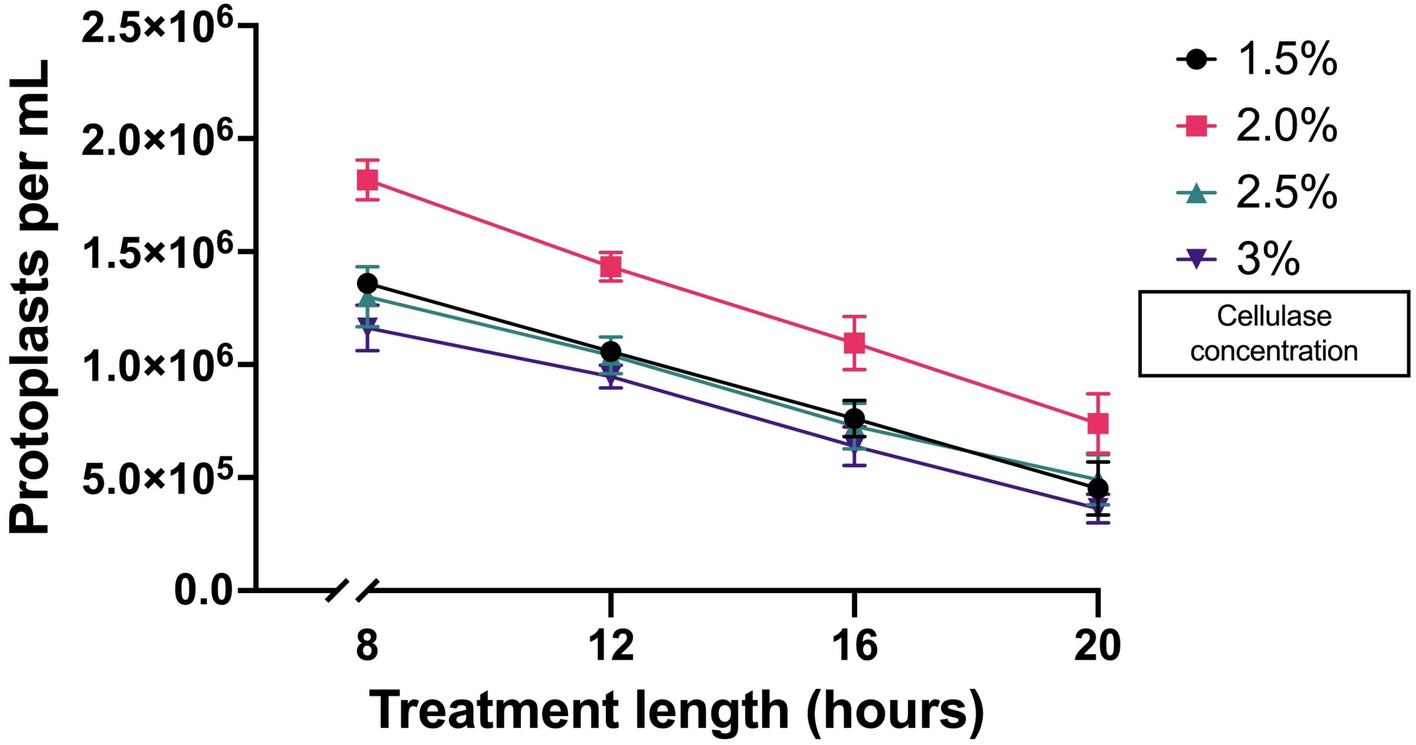
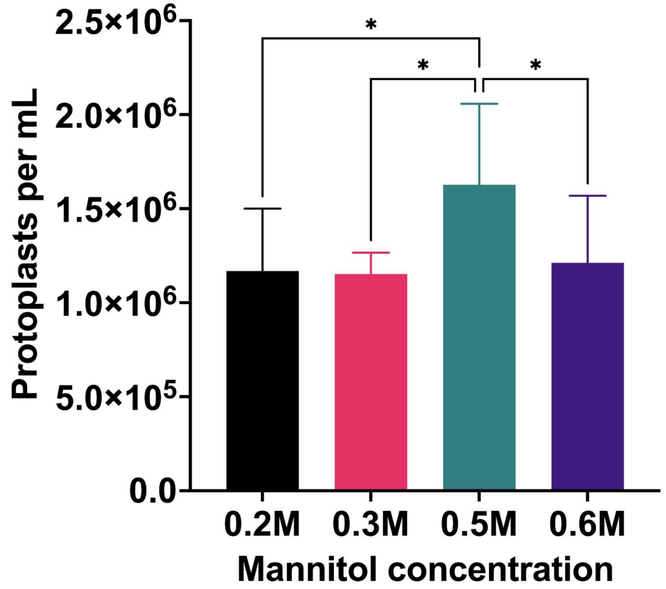
Functional characterization of frost and drought candidate genes in perennial ryegrass by CRISPR-Cas9 (Coordinator: TalTech; Involved partners: LU, NMBU)

- Candidate gene identification through bioinformatic analyses of available perennial ryegrass genome sequences
- Development of genotype-specific *in vitro* culture protocols and investigation on the most appropriate genome-editing plasmids
- 14 ecotypes/genotypes of *L. perenne* were obtained from LAMMC and NMBU. Those were selected according to variable tolerance to frost and drought
- A protocol to generate, in an asexual manner, *L. perenne* that can be used for gene editing was established. *in vitro* culture of tillers was set up and also generation of calli from the shoot apical meristematic region of the tillers
- Vernalization protocols were developed for some genotypes to promote flowering (frost tolerance phenotyping)
- Extensive optimization of protoplast protocols was conducted and their use for evaluation of genome editing efficiency was established
- Genome editing plasmid constructs for VIN3, CBP20 and CBP60g genes have been obtained and ready for Agrobacterium-mediated transformation of calli

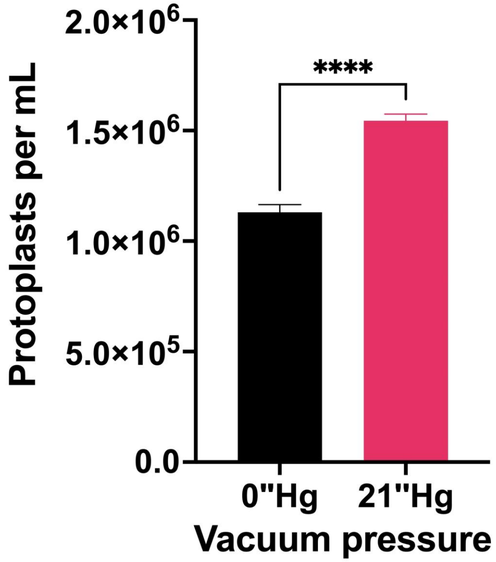
Viable protoplasts after enzymatic treatment



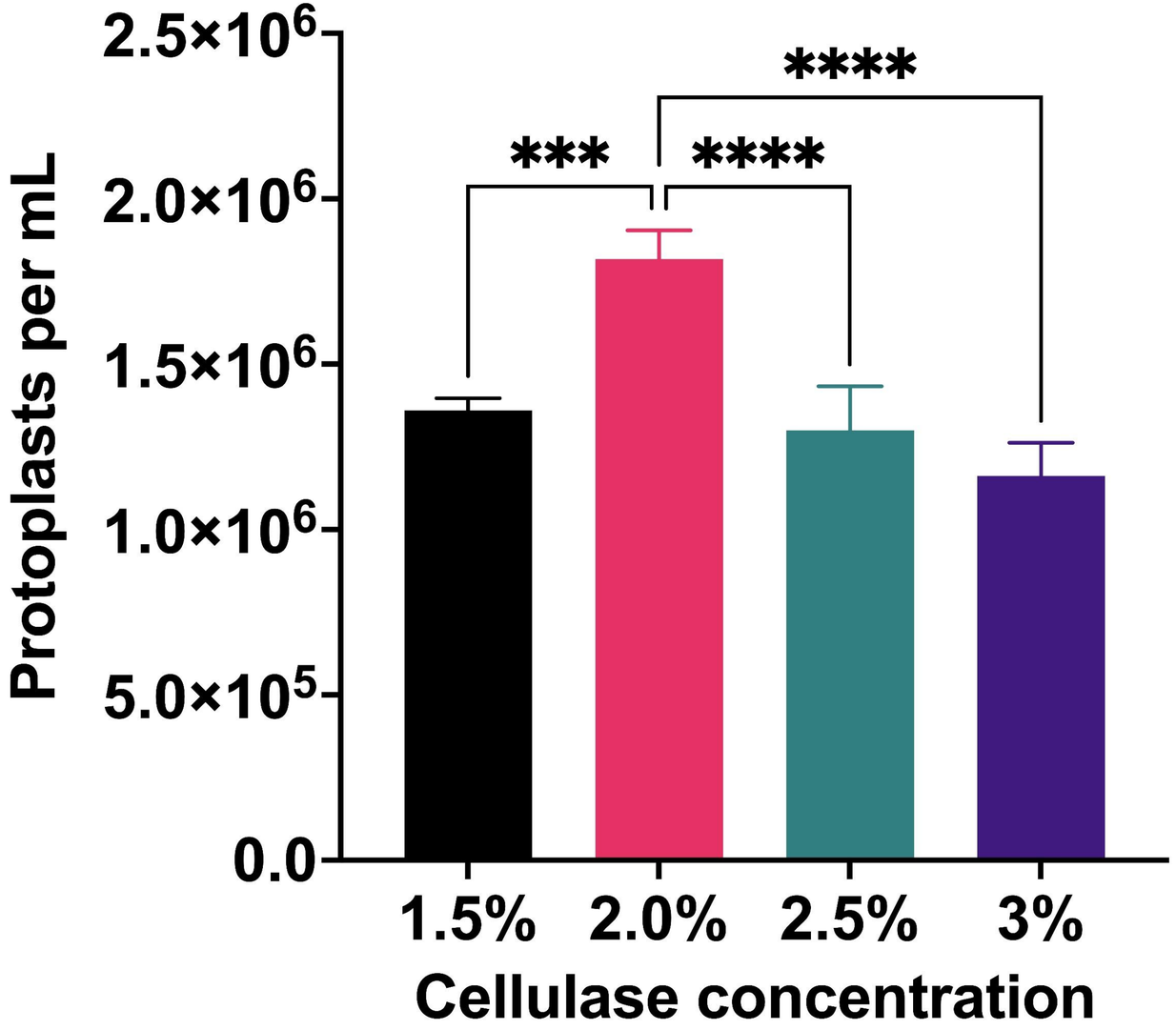
Viable protoplasts using different mannitol pretreatments

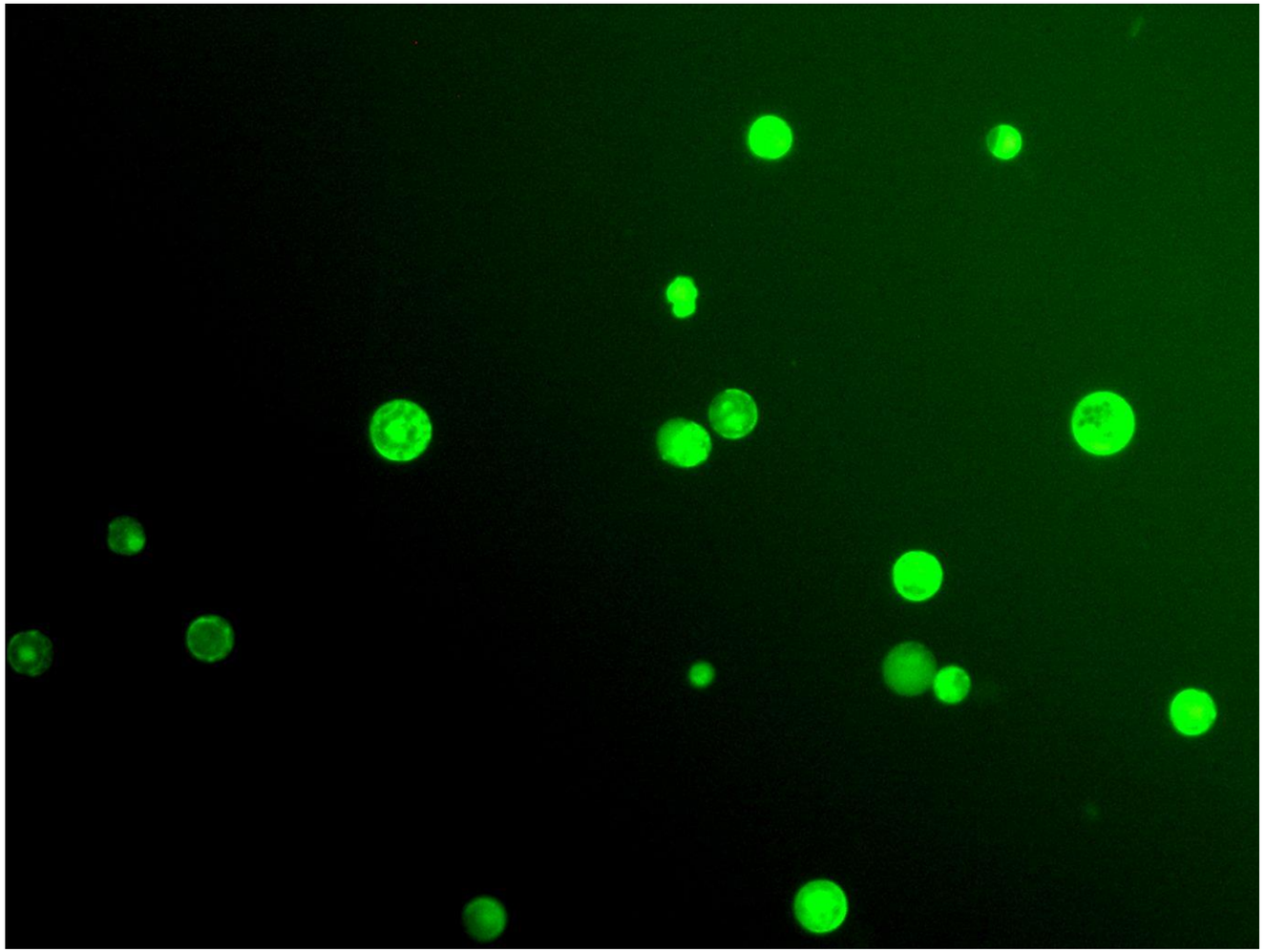


Viable protoplasts after vacuum treatment

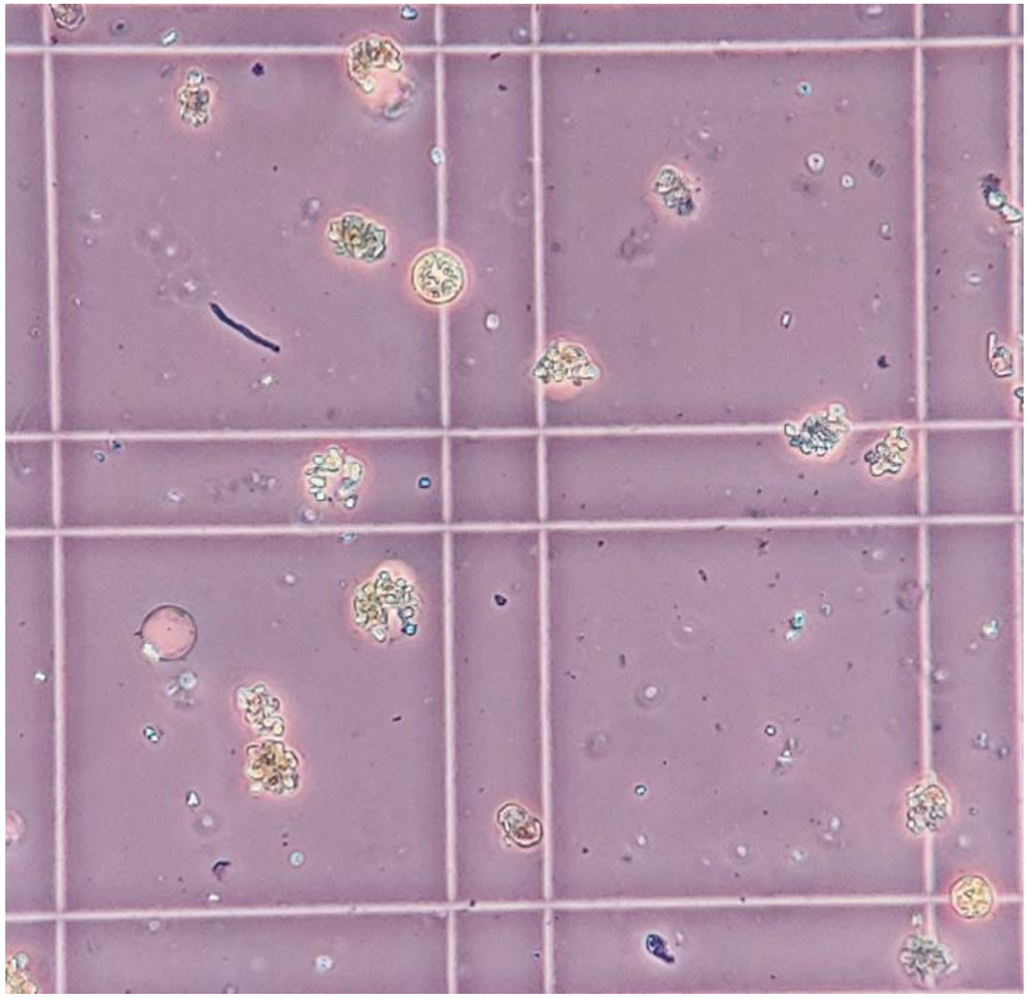


Viable protoplasts using different cellulase concentrations (8h treatment)





Fluorescein diacetate (FDA)



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Current progress of project activities – WP3

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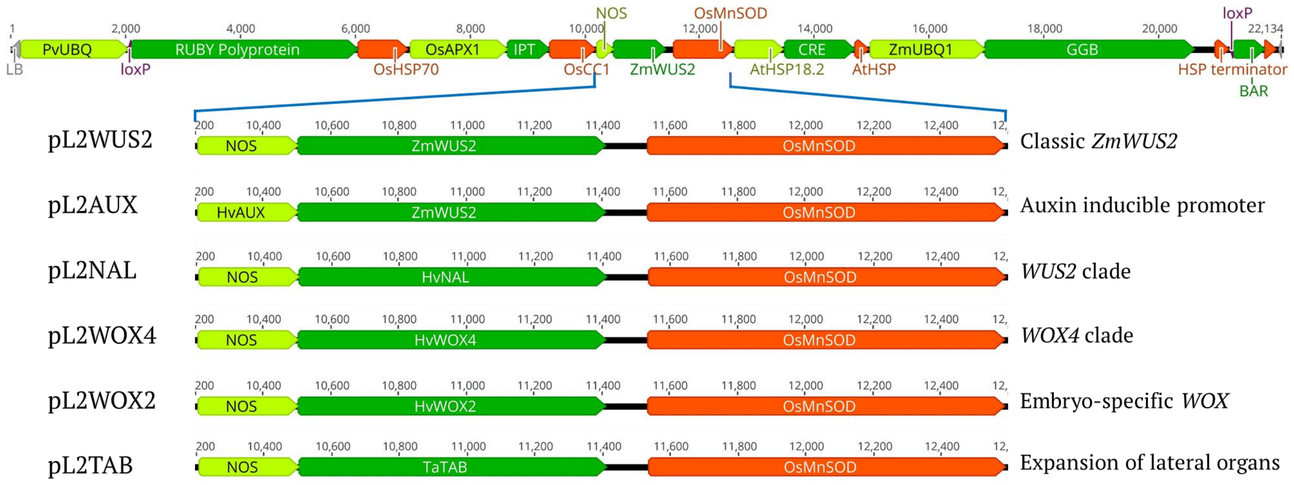
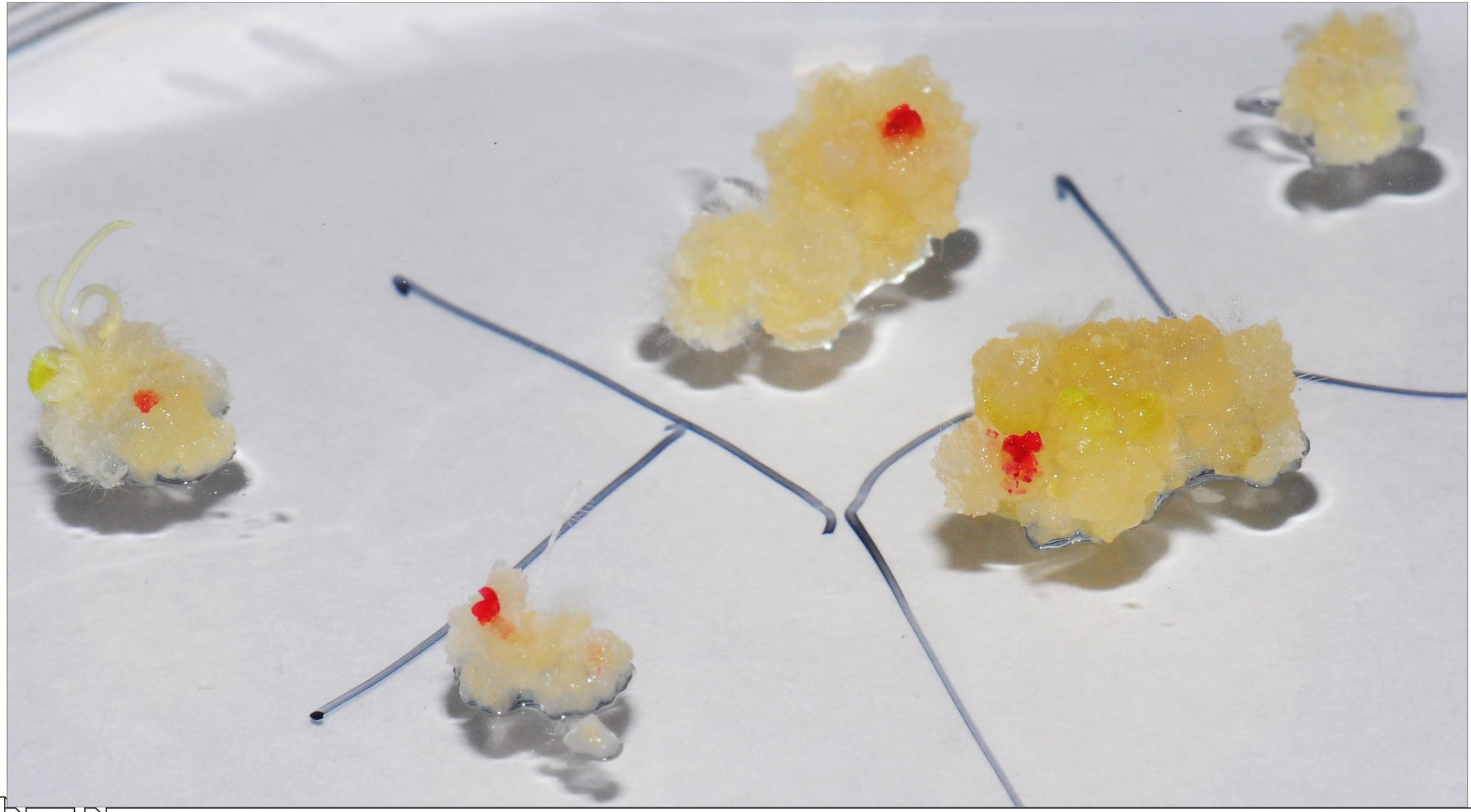
Validation of improved freezing and water shortage tolerance (Coordinator: LAMMC; Involved partners: TalTech, NMBU, LU)

- Plasmid construction for genome editing

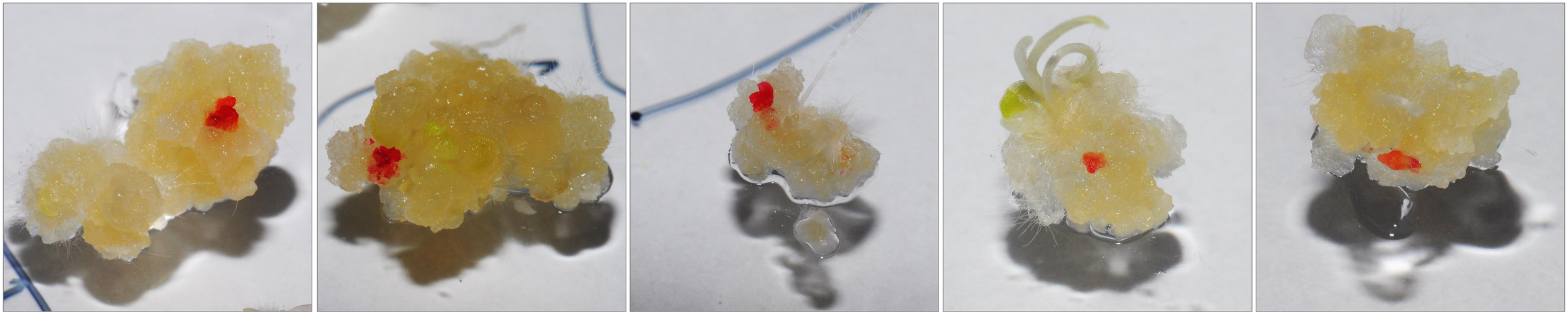


Ryegrass RUBY-positive clones produced with the T-DNA of pL2WOX4



Ryegrass SSDX3 calli were mixed with *Agrobacterium tumefaciens* (pL2WOX4) on September 21st

RUBY-positive clones show organized differentiating cellular structures



Close-ups of the ryegrass RUBY-positive clones

Current progress of project activities – WP4

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Validation of improved freezing and water shortage tolerance (Coordinator: LAMMC; Involved partners: TalTech, NMBU, LU)

- WP4 is scheduled to start in 2023, when genome edited plant are obtained
- Adjustments to phenotyping protocols

Current progress of project activities – WP5

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Management and coordination of research activities and dissemination of results (Coordinator: LU; Involved partners: TalTech, NMBU, LAMMC)

Project meetings:

- Kick-off meeting in Riga – October 7 – 8 2021 public event, steering committee meeting, internal progress report
- Annual project meeting – October 27 – 28 2022 in Tallinn public event, steering committee meeting, internal progress report
- Extra project meeting – March 30 – 31 2023 in Riga steering committee meeting and internal progress report
- Annual project meeting – October 26 - 27 2023 at LAMMC public event, steering committee meeting, internal progress report
- Final project conference – April 2024 in Riga, publick event
- Online project meetings

Current progress of project activities – WP5

EEA-RESEARCH-64

Management and coordination of research activities and dissemination of results (Coordinator: LU; Involved partners: TalTech, NMBU, LAMMC)

Project publications:

- Book chapter in 34th Meeting of the EUCARPIA Fodder Crops and Amenity Grasses (LAMMC only)
- Review article - Sustek-Sánchez F, Rognli OA, Rostoks N, Sõmera M, Jaškūnė K, Kovi MR, Statkevičiūtė G, Sarmiento C (2023) Improving abiotic stress tolerance of forage grasses – prospects of using genome editing. Frontiers in Plant Science 14
- Research article under preparation – protoplast protocols
- Publication plan established

Current progress of project activities – WP5

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Management and coordination of research activities and dissemination of results (Coordinator: LU; Involved partners: TalTech, NMBU, LAMMC)

Conferences:

- 2nd PlantEd Conference (COST Action CA18111) in Lecce, Italy, 20-22 September 2021 (Cecilia Sarmiento)
- Mendel Early career symposium in Viena, Austria May 2022 (Ferenz Sustek)
- 100th Anniversary of Plant Breeding in Lithuania conference in Akademija, Lithuania, 8 – 9 June (Nils Rostoks)
- FEBS3+ conference in Tallinn, Estonia 15 – 17 June 2022 (Nils Rostoks)
- 3rd PlantEd Conference (COST Action CA18111) in Dusseldorf, Germany, 5 – 7 September 2022 (Cecilia Sarmiento)
- EUCARPIA Fodder Crops and Amenity Grasses Conference in Brno, Czech Republic, September 10-14, 2023 (Ferenz Sustek)
- 4th PlantEd Conference (COST Action CA18111) in Porto, Portugal, 18 – 20 September 2023 (Cecilia Sarmiento, Nils Rostoks)
-

Current progress of project activities – WP5

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Management and coordination of research activities and dissemination of results (Coordinator: LU; Involved partners: TalTech, NMBU, LAMMC)

Student theses:

- Anneta Kļujeva (University of Latvia), bachelor thesis «Preparation and functional analysis of plasmid constructs for genome editing of abiotic stress tolerance candidate gene in *Lolium perenne*»
- Mari Talgø Syvertsen (Norwegian University of Life Sciences), master thesis «Establishing efficient transformation technologies for CRISPR/Cas9 genome editing in *Lolium perenne* L.»

Current progress of project activities – WP5

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Management and coordination of research activities and dissemination of results (Coordinator: LU; Involved partners: TalTech, NMBU, LAMMC)

Publicity and social media:

- Project website https://www.editgrass4food.lu.lv/en/
- Twitter - @foodedit
- ResearchGate
- Interview for the Norwegian TV (Odd Arne Rognli)

Main results achieved – based on indicators

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Indicators	Achieved/planned
Researchers (8 PhD, 4 PhD students) supported	13 / 12
Joint peer-reviewed scientific publications	1 / 4
Joint applications for further funding	0 / 1
Jointly registered applications for Intellectual Property Protection	0 / 0

Lessons learned from implementation

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- COVID – 19 pandemic (problems related to travel, attending conferences, as well as increased delivery times for consumables)
- Difficulties to recruit PhD students (have been solved, but caused delay in the project)
- Research challenges:
  - Difficulty to obtain and propagate perennial ryegrass *in vitro* cultures for AMT
  - Difficulty to identify and re-sequence abiotic stress candidate genes (highly heterozygous genome)
  - Ryegrass genotypes recalcitrant to AMT and regeneration

Added value of programme

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Benefits from the implementation of the project:

- Collaboration among project partners
- Shared plant material, gene sequences, plasmid constructs etc.
- Possibilities for training students

Iceland
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grants

QUESTIONS?

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