Improving perennial ryegrass adaptability and resilience (EditGrass4Food)

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The aim of the project is to improve adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR-Cas9 technology. Genes involved in the mechanisms of freezing tolerance and biomass growth under water deficit will be investigated and targeted via gene editing.
"EditGrass4Food" is a EEA-Norway Grant (Baltic Research Program) project that has started this year and is coordinated by the University of Latvia.
Perennial ryegrass (*Lolium perenne*) is the dominant forage grass species in Europe. However, perennial ryegrass exhibits poor performance under unfavourable environmental conditions, thus the changing climate poses a substantial challenge to ryegrass cultivation in the Baltic/Nordic region. In this project, we intend to utilize unique pre-breeding material, developed by the members of our consortium and CRISPR-based editing to validate candidate genes involved in northern adaptation of perennial ryegrass. We will investigate changes during abiotic stress periods at the transcriptome level to reveal gene regulatory pathways. Improving perennial ryegrass for winter hardiness, persistence and biomass formation under water limited conditions will help breeders in the Nordic/Baltic region to prepare for meeting new demands due to climate change.

Iceland Liechtenstein Norway grants

PARTNERS:

Tallinn University of Technology

Lithuanian Research Centre for Agriculture and Forestry

Norwegian University of Life Sciences

University of Latvia (coordinator)

Main objectives:

1. Establish a diverse perennial ryegrass core association panel by utilization of data from ongoing projects.
2. Screen the association panel to detect haplotype-resolved single-nucleotide variants and structural variation in the targeted genes/alleles for freezing and drought genes.
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.
4. Develop CRISPR-Cas9 constructs and generate CRISPR-edited perennial ryegrass mutants for freezing and mild drought tolerance.
5. Validate and characterize the role of the genes and their sequence variations in the freezing and drought mechanisms.

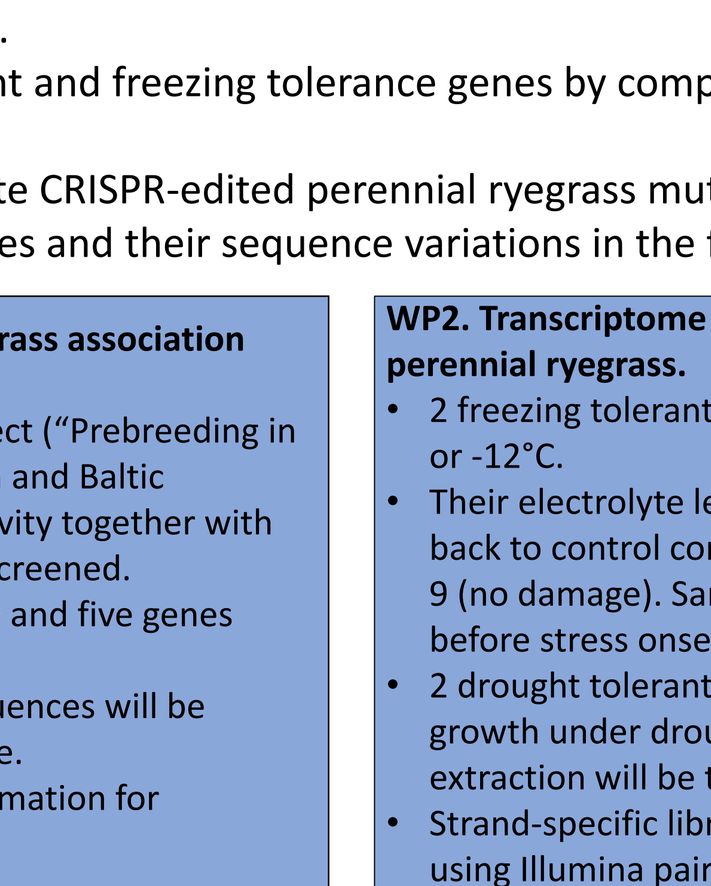
WP1. Establishment and screening of perennial ryegrass association panel for freezing and drought related traits.

- Genotypes, from plants grown under the PPP project ("Prebreeding in perennial ryegrass project's" partners: all Northern and Baltic countries) that show freezing tolerance and sensitivity together with genotypes that present drought tolerance will be screened.
- Five candidate genes involved in freezing tolerance and five genes related to drought resistance will be selected.
- PromethION sequencing will be used, and the sequences will be aligned to the perennial ryegrass reference genome.
- Functional SNPs identified will be used as key information for identification of the targets for CRISPR.

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

- 2 freezing tolerant and 2 susceptible genotypes subjected to freezing at -8 or -12°C.
- Their electrolyte leakage will be measured, and plants will be transferred back to control conditions and scored for freeze damage from 0 (dead) to 9 (no damage). Samples of leaf will be taken for RNA extraction at the day before stress onset and 8 hours after the freezing exposition.
- 2 drought tolerant and 2 susceptible genotypes will be measured for leaf growth under drought conditions for 5 days and leaf samples for RNA extraction will be taken every day.
- Strand-specific libraries will be created and sequenced across all lines using Illumina paired-end sequencing.

Forage production from grasslands represents locally produced feed resources for ruminant livestock production. In all European regions, grassland systems and forage crops will have to use nutrients and water more efficiently to maximize production per unit area and adapt to the UN SD goals. Global warming is expected to increase productivity in Northern Europe. The higher temperatures, changes in precipitation patterns, and higher frequency of extreme climatic events create a greater incidence of drought. *Lolium perenne* has superior feed quality and productivity, however, it is not well adapted to the Nordic-Baltic weather.


Perennial ryegrass field at Graminor, Norway

Potential candidate genes for

drought tolerance:
PHYB
MYB41
NAC038
MYB94
WSD11
TSO1
MYB4
HSL1
WRKY49
PRR5

frost tolerance:
VIN3
QM
TPT
IRI1
CBF6
DHN1
GI
LEA-14
PRR95
EF-CAX

WP3. Functional characterization of frost and drought candidate genes in perennial ryegrass by CRISPR-Cas9.

- 4 candidate genes for freezing and drought related traits will be knocked-out using the CRISPR-Cas9 technology.
- Transient protoplast assays, using *Agrobacterium*-mediated transformation, will allow to test the efficiency of designed sgRNAs to target candidate genes.
- Protocols for selecting edited embryogenic callus cultures without marker genes will be developed using PCR tests and sequencing.
- Regeneration protocols from embryogenic calli will be optimized for selected *Lolium* genotypes. Regenerated *Lolium* plants will then be cloned and used for drought and frost tolerance tests.

WP4. Validation of improved freezing and water shortage tolerance.

- Validation and characterization of the role of the genes involved in tolerance to freezing and drought by phenotyping plants under controlled environment.
- 10 gene-edited plants will be cold-acclimated and tested for freezing tolerance. After freeze test, plants will be removed from the freezing chamber and thawed on ice overnight at 2 °C. Tillers will be transplanted into soil and grown for 3 weeks before being scored for survival.
- Leaf growth under water deficit regime will be used to assess the tolerance to drought of 10 gene-edited plants.

Through the Nordic-Baltic PPP project on perennial ryegrass, genetic resources and new breeding materials have been studied and created to help adapt this species to the northern region conditions. This project will allow designing better breeding schemes for perennial ryegrass, and therefore cultivars with improved climate adaptation more rapidly.
The Baltic region is lagging in the field of the innovative biotechnology industry, especially in agriculture. The project expects to help solve this delay by developing a CRISPR/Cas genome editing system for perennial ryegrass. Fostering the breeding of freezing and drought-tolerant genotypes will shorten the breeding of new cultivars thus lowering its costs and making it more competitive on the market. Additionally, varieties with a wide genetic plasticity and adaptation capacity would have higher water and nitrogen use efficiency, thus contributing to safer and higher quality feed production.
(Ferenz Sustek Sánchez - PhD student working in this project - helped with this poster).