Iceland Liechtenstein Norway grants

EDIT GRASS 4FOOD

IMPROVING ADAPTABILITY AND RESILIENCE OF PERENNIAL RYEGRASS FOR SAFE AND SUSTAINABLE FOOD SYSTEMS THROUGH CRISPR-Cas9 TECHNOLOGY – EditGrass4Food

The project aims 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. This is one of the Baltic Research Programme's projects and is financially supported by the European Economic Area (EEA) grants with 1 million euros.

Working together for green, competitive and inclusive Europe

Climate change is expected to have a negative impact on agriculture and food security. The European Green deal and farm to Fork (F2F) Strategy envisage that biotechnology will play an important role in ensuring food safety and security. Contributing to sustainable development of the EU economy. Grasslands are essential for the production of animal feed, although – they also serve as carbon sink and provide culturally important landscapes. Perennial ryegrass *(Lolium perenne L.)* is an important plant species in meadows and pastures, but it often lacks the resilience to climatic conditions in northern Europe and the Baltics, where weather conditions are becoming increasingly unpredictable due to climate change. The EditGrass4Food project brings together leading scientists from the Baltic States and Norway to develop solutions to the complex problem of abiotic stress as a result of climate change, using the important forage grass species perennial ryegrass as a model. **The main goal of the project was to characterize the abiotic stress tolerance in a set of genotypes of perennial ryegrass, to assess their genetic basis by studying changes in gene expression, as well as to improve abiotic stress tolerance by editing key genes using the CRISPR/Cas9 system.** Project results will provide forage and grassland plant breeders with useful information on genotypes with better abiotic stress tolerance, as well as specific target genes involved in these processes.

Abiotic stress phenotyping and transcriptome analysis in perennial ryegrass

The set of 160 perennial ryegrass genotypes were selected based on their freezing tolerance studied previously. Each genotype was vegetatively propagated and planted in cell packs filled with peat substrate, with 4 ramets per cell and grown until full establishment. Cold acclimation was performed in the growth chambers at 5 °C for 7 days, continued at 2 °C for 14 days. The acclimated plants were further used for freezing experiments where freezing tolerance was defined as electrolyte leakage (EL) in leaf tissue. The freezing test was conducted at the targeted temperature of -12 ^{0}C and -14 ^{0}C. The design of the experiment is presented in Fig. 1. For EL estimation, leaf samples of each genotype in 4 replicates were placed in the tubes, filled with 2 ml of distilled water and moved into the climate chamber where freezing tests were carried out as described below. Electrolyte leakage was estimated as EL = (C_{ini})/(C_{tot}) x 100, where initial conductivity (C_{ini}) was measured after shaking tubes overnight and total conductivity (C_{tot}) was measured after autoclaving the samples at 120 °C. Substantial variation for freezing tolerance parameters was observed among the tested genotypes and ranged from 9.74 to 61.0% with a mean of 31.3%. The observed results allowed us to identify two of the most freezing tolerant and two the most sensitive genotypes which were used for transcriptome studies (Fig. 2). The selected genotypes were vegetatively propagated into 20 replicates, planted in a peat substrate, and acclimated as described above and freeze tested at a target temperature of –14 °C held for 24 h. The crown tissues of each genotype in three replicates were collected at six times points (Fig. 1) and immediately frozen in liquid nitrogen for further RNA extractions.

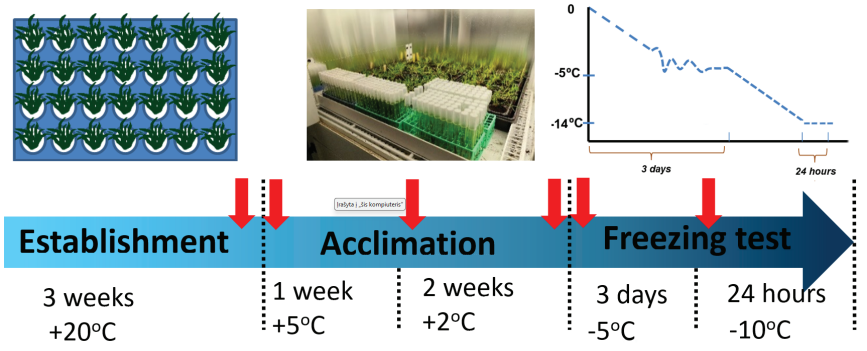

Fig. 1

The design of cold acclimation and freezing experiment with indicated time point (red arrows) of RNA sampling

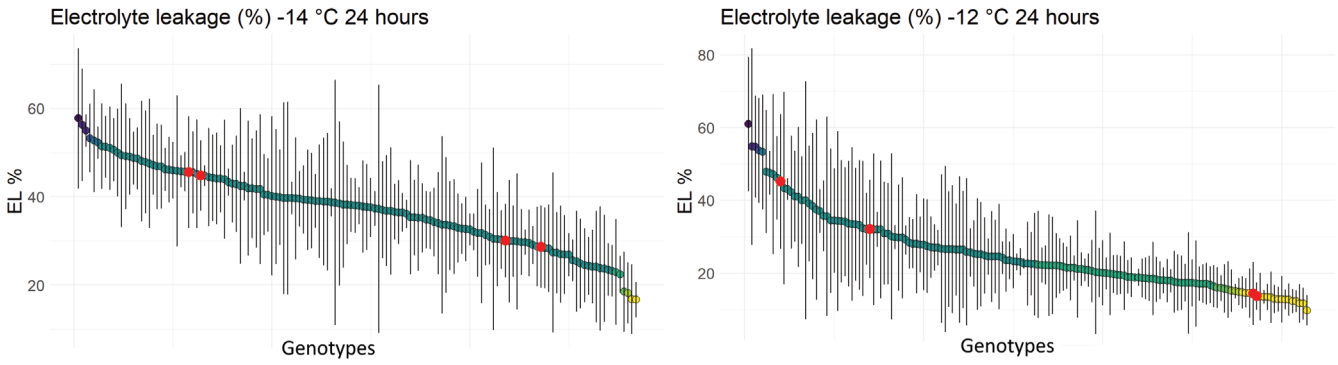

Fig. 2

Electrolyte leakage (EL) variation among perennial ryegrass genotypes, where A represents the EL results after freezing at -14°C, and B – at -12°C, while error bars represent standard deviation (SD).The genotypes selected for the transcriptome analysis are shown as red dots.

Development of genome editing platform in perennial ryegrass

The ambitious goal of achieving increased stress tolerance through genome editing in ryegrass started the development of an efficient genome editing platform for the species. As a highly diverse outbreeding monocot species perennial ryegrass is a challenging object for plant tissue culture-based methods, Agrobacterium mediated transformation and whole-plant regeneration. A two-step approach was adopted to achieve genome-edited *L. perenne* -first, the preliminary testing of selected protospacers of *L. perenne* target genes was done in the protoplast system, second, the development of genome-edited plants was achieved through callus transformation and plant regeneration. In addition, the problem of *L. perenne* recalcitrance to tissue culture was addressed by the project activity investigating the ectopic expression of morphogenic regulators which improve regeneration of the transformed calli.

The development of the method for the protoplast isolation from *L. perenne* leaves resulted in a robust protocol allowing for protoplast yields sufficient for transformation and protospacer efficiency testing (Fig. 3).

The production of genome-edited plants was achieved via stable Agrobacterium mediated transformation of shoot-tip culture derived calli and a subsequent plant regeneration. The design of constructs for *L. perenne* genome editing was based on the golden-gate approach and custom made vector. To relieve the selection process of transformed calli at early stages of transformation, the reporter gene ZSGreen was introduced in T-DNA cassettes in addition to the original selection marker - hygromycin resistance (Fig. 6). Simplified identification of transformation events in combination with targeted selection of most proliferating callus lines allowed for establishment of transformed regenerated plants within a comparatively short period - 48 days (Fig. 4,5).

Heterologous expression of morphogenic regulators GRF-GIF-BBM and WUS of different taxonomic origin in *L. perenne* resulted in morphological traits indicating increased pluripotency of meristematic cells and relieved tissue culture recalcitrance (Fig. 8, Fig. 9). In addition, RUBY worked as a valuable reporter gene for the selection of successful transformation events in *L. perenne* (Fig. 7).

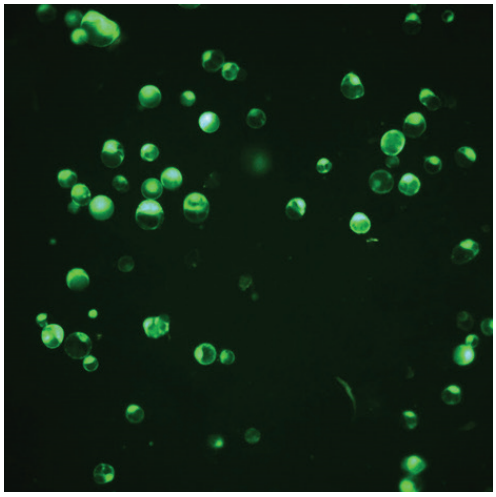

Fig. 3

L. perenne protoplasts stained with fluorescein diacetate for viability testing

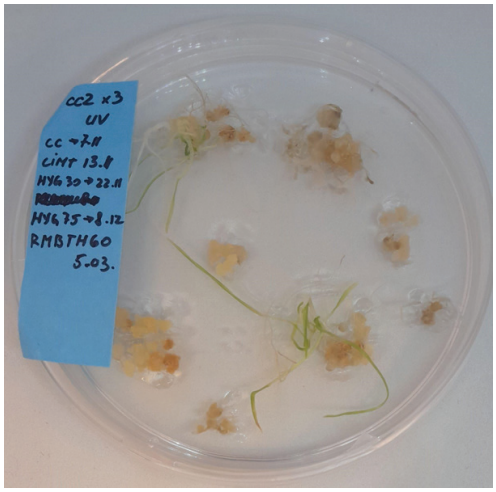

Fig. 4

Regeneration of transformed L. perenne calli – emerging shoots

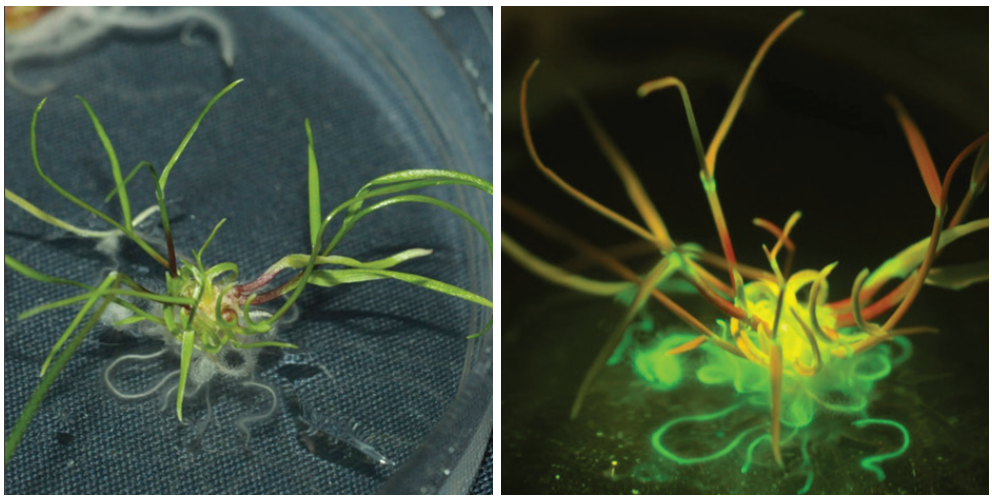

Fig. 5

Regenerated L.perenne transformant in visible light (*left*) *and in blue* (*right*)

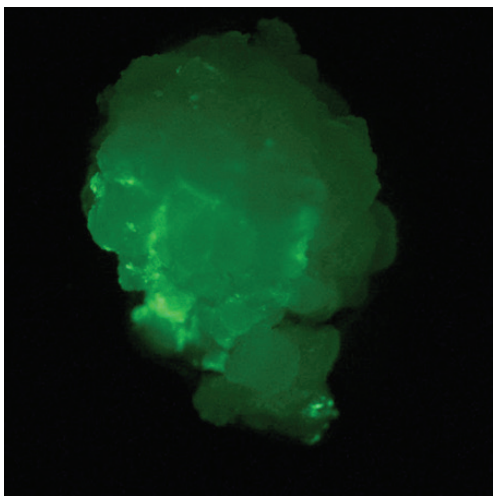

Fig. 6

L. perenne callus expressing visual selection marker ZSGreen

Fig. 8

L. perenne root cultures of low (left) and high (right) ectopic expression of morphogenic regulator BBM and visual selection marker RUBY

Fig. 7

L. perenne callus expressing visual selection marker RUBY in successfully transformed cells

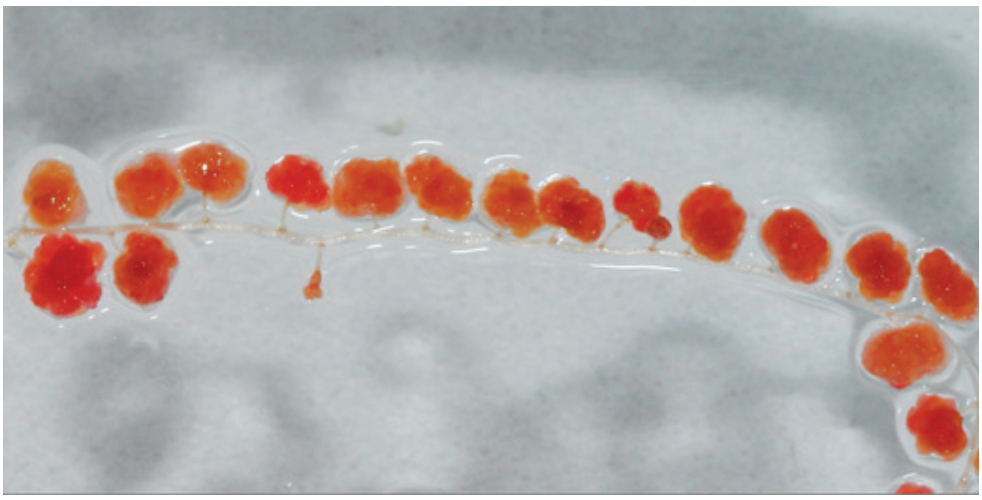

Fig. 9

Morphology of L. perenne root ectopically expressing morphogenic regulator BBM and visual selection marker RUBY

The project is developed in cooperation with the University of Latvia, Norwegian University of Life Sciences, Lithuanian Research Centre for Agriculture and Forestry, and Tallinn University of Technology

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