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

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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/

Perennial ryegrass

- *Lolium* perenne is an important forage grass species widely cultivated in Europe, N. America, N. Zealand
- high yielding, excellent forage quality and palatability
- *L. perenne* is an outcrossing, wind-pollinated species
- selfing is largely prevented by a gametophytic, two-locus incompatibility system (*SZ*)
- originates from Mediterranean region
- Lacks adaptation to Nordic-Baltic climate conditions

Perennial ryegrass

- Model plant, diploid, rather small genome
- Genome is heterozygous and the varieties consist of a mixture of related genotypes
- Genotypes exhibit different efficiciency of *Agrobacterium*-mediated transformation (CRISPR/Cas constructs) and variable regeneration capacity

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SEB Society for Experimental Biology

The Plant Journal (2015) **84**, 816–826 doi: 10.1111/tpj.13037

RESOURCE

A synteny-based draft genome sequence of the forage grass *Lolium perenne*

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Received 22 July 2015; revised 4 September 2015; accepted 18 September 2015; published online 26 September 2015.
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SUMMARY

Here we report the draft genome sequence of perennial ryegrass (*Lolium perenne*), an economically important forage and turf grass species that is widely cultivated in temperate regions worldwide. It is classified along with wheat, barley, oats and *Brachypodium distachyon* in the Pooideae sub-family of the grass family (Poaceae). Transcriptome data was used to identify 28 455 gene models, and we utilized macro-co-linearity between perennial ryegrass and barley, and synteny within the grass family, to establish a synteny-based linear gene order. The gametophytic self-incompatibility mechanism enables the pistil of a plant to reject self-pollen and therefore promote out-crossing. We have used the sequence assembly to characterize transcriptional changes in the stigma during pollination with both compatible and incompatible pollen. Characterization of the pollen transcriptome identified homologs to pollen allergens from a range of species, many of which were expressed to very high levels in mature pollen grains, and are potentially involved in the self-incompatibility mechanism. The genome sequence provides a valuable resource for future breeding efforts based on genomic prediction, and will accelerate the development of new varieties for more productive grasslands.

Keywords: ***Lolium perenne*, perennial ryegrass, genome sequence, self-incompatability, pollen allergens.**

INTRODUCTION

Ryegrasses (*Lolium* spp.) and fescues (*Festuca* spp.) are the principle forage grasses underpinning forage-based meat and dairy production throughout the temperate world. The *Lolium* genus consists of nine closely related species that share a close evolutionary relationship to a number of broad-leaf fescues (sub-genus *Schendonorus*, also frequently referred to as *Festuca* spp.). Species within the *Lolium*/*Festuca* complex are partially interfertile, form a well-defined ploidy series, and incorporate a wide range of variation in terms of phenology, agronomy and specific adaptive traits (Humphreys *et al.*, 2006). Perennial ryegrass (2*n* = 14) has particular importance as a forage grass in temperate climate zones, and may be utilized as hay, silage and pasture. Its agricultural value lies in its rapid establishment, high yields, long growing season, tolerance of grazing, and high palatability and digestibility for ruminant

Plant Biotechnology Journal

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Plant Biotechnology Journal (2020) **18**, pp. 1854–1856 doi: 10.1111/pbi.13359

Brief Communication

Targeted mutagenesis in ryegrass (*Lolium* spp.) using the CRISPR/Cas9 system

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Received 15 December 2019;
revised 4 February 2020;
accepted 9 February 2020.
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Keywords: ryegrass, CRISPR/Cas9, DMC1.

Ryegrass is one of the most important forage crops worldwide. It is the basis for 80% of milk production and 70% of meat production and has major economic importance. Breeding programmes for ryegrass started in the 1920s, and breeders have mainly relied on repeated phenotypic and recently genotypic selection of elite individuals. Although this approach has led to significant improvements in several characters including rust resistance, spring growth and aftermath heading, it tends to be laborious, expensive and time-consuming, mainly due to gametophyte self-incompatibility in most ryegrass species (Sampoux *et al.*, 2011). In order to overcome some of the limitations of traditional introgression and selective breeding, modern methods of mutation induction offer attractive opportunities to target specific genes of interest and directly introduce allelic variability. In the last decade, the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated endonuclease 9 (CRISPR/Cas9) system has been extensively used in most crops and is paving the way to precision trait improvements in factors including yield, quality, biotic- and abiotic stress resistance and breeding rate (Chen *et al.*, 2019; Ran *et al.*, 2017; Wang *et al.*, 2014). However, this powerful tool for genome editing has not yet been used in ryegrass. Meiosis arose early during the evolution of eukaryotes and is vital for sexual reproduction, not only in relation to genomic stability but also to genetic diversity. Meiotic studies of plants in the areas of crop fertility and genetic variation have important potential agronomical applications. *DMC1 (DISRUPTED MEIOTIC cDNA1)*, initially identified in yeast (Bishop *et al.*, 1992) as a homolog of the bacterial strand exchange protein RecA, is a crucial meiotic recombinase. Here, we describe the use of the CRISPR/Cas9 system to introduce mutations in *LpDMC1* in two species: Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) and perennial ryegrass (*Lolium perenne*). We succeeded in obtaining both T0 homozygous and heterozygous mutants, and the T0 null mutants of Italian ryegrass exhibited complete male sterility and severely disordered meiosis with univalents and multivalents appearing at diakinesis.

To see whether mutations could be introduced into ryegrass using the CRISPR/Cas9 system, we generated a sgRNA (*TS-LpDMC1)* targeting exon 5 of *LpDMC1*, with an *Xcel* restriction enzyme site near the protospacer-adjacent motif (PAM) for ease of analysis (Figure 1a). Because plant tissue culture and genetic transformation are time-consuming, we tested the activities of sgRNA in a protoplast transient expression system as described by Shan *et al.* (2013). *TS-LpDMC1* promoted by TaU6 was co-introduced with SpCas9 into ryegrass protoplasts by PEG-mediated transformation. After 40- to 48-h incubation, analysis of genomic DNA using a PCR restriction enzyme digestion assay (PCR/RE) demonstrated the occurrence of indel mutations at the target site (Data not shown).

To determine whether the CRISPR/Cas9 method was applicable to other ryegrass genes, we targeted the ryegrass orthologue of centromere-specific histone H3 variant (CENH3). In *Arabidopsis thaliana*, CENH3 ensures faithful transmission of the genome at cell division, and when *cenh3* null mutants producing altered CENH3 proteins are crossed with wild type, many haploid *Arabidopsis* plants are generated (Ravi and Chan, 2010). When we co-transformed a sgRNA targeting exon 3 of *LpCENH3* along with SpCas9 into protoplasts (Figure 1d), PCR/RE analysis revealed frameshift mutations at the target site (Figure 1e). These results show that CRISPR/Cas9 can be used to generate mutations in ryegrass.

Next, the sgRNA expression cassette was combined with SpCas9 in a single DNA construct by GIBSON Assembly and introduced along with a hygromycin-resistant plasmid into preconditioned embryogenic callus (EC) lines of ryegrass by gold particles bombardment. To generate these EC lines, seeds of Italian ryegrass cultivar Gepetto and perennial ryegrass cultivar Goyave were de-husked and sterilized, and somatic EC lines were established as described (Ran *et al.*, 2014). Three separate lines designated Gepetto-8, Gepetto-66 and Goyave LMG LDF-Lp3711 (provided by DLF Seeds) with outstanding regeneration ability were selected for transformation. After bombardment, the EC was transferred to hygromycin medium. Surviving calli were obtained after 4 weeks' induction and sub-culture. Thereafter, they were regenerated for 8 weeks; and plantlets with established roots were transferred to potting mix for mutants' identification. The mean transformation efficiencies of these three lines were 3.83%, 4.50% and 2.66%, respectively. The entire experimental cycle took approximately 10 months from

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Ultralong Oxford Nanopore Reads Enable the Development of a Reference-Grade Perennial Ryegrass Genome Assembly

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Accepted: 2 July 2021

Abstract

Despite the progress made in DNA sequencing over the last decade, reconstructing telomere-to-telomere genome assemblies of large and repeat-rich eukaryotic genomes is still difficult. More accurate basecalls or longer reads could address this issue, but no current sequencing platform can provide both simultaneously. Perennial ryegrass (*Lolium perenne* L.) is an example of an important species for which the lack of a reference genome assembly hindered a swift adoption of genomics-based methods into breeding programs. To fill this gap, we optimized the Oxford Nanopore Technologies' sequencing protocol, obtaining sequencing reads with an N50 of 62 kb—a very high value for a plant sample. The assembly of such reads produced a highly complete (2.3 of 2.7 Gb), correct (QV 45), and contiguous (contig N50 and N90 11.74 and 3.34 Mb, respectively) genome assembly. We show how read length was key in determining the assembly contiguity. Sequence annotation revealed the dominance of transposable elements and repeated sequences (81.6% of the assembly) and identified 38,868 protein coding genes. Almost 90% of the bases could be anchored to seven pseudomolecules, providing the first high-quality haploid reference assembly for perennial ryegrass. This protocol will enable producing longer Oxford Nanopore Technology reads for more plant samples and ushering forage grasses into modern genomics-assisted breeding programs.

Key words: *Lolium perenne*, forage grasses, perennial ryegrass, genomics, genome assembly, Oxford Nanopore.

Significance

Sequencing eukaryotic genomes with long-read sequencing platforms is allowing to obtain genome assemblies of unprecedented quality also for many non-model organisms. However, especially in genomes with a high amount of long repeats, completeness and contiguity are limited by the quality (accuracy and/or length) of the input data. Here we present an innovative protocol for Oxford Nanopore Technologies' genomic plant DNA library preparation that considerably increases read length. We show how these exceptionally longer reads were key in obtaining a perennial ryegrass genome assembly with unprecedented statistics, both within its genus and among other plants of similar complexity. This work makes available a highly complete and contiguous genome assembly and the laboratory protocol necessary to produce long read data.

Nagy *et al. BMC Genomics* (2022) 23:505
https://doi.org/10.1186/s12864-022-08697-0

BMC Genomics

RESEARCH Open Access

Chromosome-scale assembly and annotation of the perennial ryegrass genome

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Abstract

Background: The availability of chromosome-scale genome assemblies is fundamentally important to advance genetics and breeding in crops, as well as for evolutionary and comparative genomics. The improvement of long-read sequencing technologies and the advent of optical mapping and chromosome conformation capture technologies in the last few years, significantly promoted the development of chromosome-scale genome assemblies of model plants and crop species. In grasses, chromosome-scale genome assemblies recently became available for cultivated and wild species of the Triticeae subfamily. Development of state-of-the-art genomic resources in species of the Poeae subfamily, which includes important crops like fescues and ryegrasses, is lagging behind the progress in the cereal species.

Results: Here, we report a new chromosome-scale genome sequence assembly for perennial ryegrass, obtained by combining PacBio long-read sequencing, Illumina short-read polishing, BioNano optical mapping and Hi-C scaffolding. More than 90% of the total genome size of perennial ryegrass (approximately 2.55 Gb) is covered by seven pseudo-chromosomes that show high levels of collinearity to the orthologous chromosomes of Triticeae species. The transposon fraction of perennial ryegrass was found to be relatively low, approximately 35% of the total genome content, which is less than half of the genome repeat content of cultivated cereal species. We predicted 54,629 high-confidence gene models, 10,287 long non-coding RNAs and a total of 8,393 short non-coding RNAs in the perennial ryegrass genome.

Conclusions: The new reference genome sequence and annotation presented here are valuable resources for comparative genomic studies in grasses, as well as for breeding applications and will expedite the development of productive varieties in perennial ryegrass and related species.

Keywords: *Lolium perenne*, Perennial ryegrass, Chromosome-scale assembly, *Festuca-Lolium* complex, Comparative genomics

Background

Grasslands make up 40 percent of the earth's temperate and tropical terrestrial surface covering an estimated total area of about 52 million km^2 [1]. Eighty percent of the world's bovine milk and seventy percent of the world's beef and veal are produced from temperate grassland systems [2]. *Lolium perenne* L. (perennial ryegrass) is one of the most important forage species for ruminant animal production in temperate regions. The *Lolium* genus consists of ten diploid species [3] that share a close evolutionary relationship to broad leaf fescues that belong to the large and diverse genus *Festuca*. The majority of species within the *Festuca-Lolium* complex are obligate outbreeders and partially interfertile, forming a well-defined ploidy series and incorporating a wide range of variation in terms of phenology, agronomy and specific adaptive traits [4].

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Rounding up the annual ryegrass genome: high-quality reference genome of *Lolium rigidum*

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Abstract

The genome of the major agricultural weed species, annual ryegrass (*Lolium rigidum*) was assembled, annotated and analysed. Annual ryegrass is a major weed in wheat cropping, and has the remarkable capacity to evolve resistance to herbicides with various modes of action. The chromosome-level assembly was achieved using short- and long-read sequencing in combination with Hi-C mapping. The assembly size is 2.44Gb with N_{50}=361.79Mb across 1,764 scaffolds where the seven longest sequences correspond to the seven chromosomes. Genome completeness assessed through BUSCO returned a 99.8% score for complete (unique and duplicated) and fragmented genes using the Viridiplantae set. We found evidence for the expansion of herbicide resistance-related gene families including detoxification genes. The reference genome assembly of *L. rigidum* is pivotal for the management of this highly problematic weed species which leverages genomic tools to devise new control options.

Introduction

Lolium rigidum (Gaudin, 1811) also known as annual ryegrass, rigid ryegrass, or Wimmera grass, is the world's most herbicide resistant weed species. It has developed resistance to over a dozen different modes of action across a number of herbicides and has the highest incidence of resistance in any weed species (Heap 2022). It is the first weed species reported to have evolved resistance to glyphosate (Powles et al. 1998).

L. rigidum is a diploid grass species with a chromosome number of 2n=2x=14 (Terrell 1966; Monaghan 1980) and an estimated genome size of ~2Gb, similar to that of the closely-related forage crop *Lolium perenne* (Byrne et al. 2015; Frei et al, 2021). This species known to hybridise with other members of the *Lolium* genus, e.g. *L. multiflorum* and *L. perenne* (Kloot 1983). This genus is thus a complex of cross-compatible species which can produce fertile hybrids and makes species boundaries ambiguous (Naylor 1960; Terrell 1966; Kloot 1983).

DISS. ETH NO. 28398

Establishing CRISPR/Cas9-mediated mutagenesis in perennial ryegrass (*Lolium perenne* L.)

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2022

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Article

Callus Induction from Diverse Explants and Genotypes Enables Robust Transformation of Perennial Ryegrass (*Lolium perenne* L.)

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Abstract: Genetic transformation of perennial ryegrass (*Lolium perenne* L.) is critical for fundamental and translational research in this important grass species. It often relies on *Agrobacterium*-mediated transformation of callus tissue. However, callus induction is restricted to a few genotypes that respond well to tissue culture. Here, we report callus induction from different perennial ryegrass genotypes and explants, such as shoot tips, seeds, and anthers, which were transformed with several plasmids for functional genomics. β-glucuronidase (GUS) histochemical staining showed the *LmdsRNAbp* promoter sequence was active in stigmas, spikelets, anthers, and leaves. We also transformed calli with plasmids allowing gene silencing and gene knock-out using RNA interference and CRISPR/Cas9, respectively, for which genotypic and phenotypic investigations are ongoing. Using 19 different constructs, 262 transgenic events were regenerated. Moreover, the protocol regenerated a doubled haploid transgenic event from anther-derived calli. This work provides a proof-of-concept method for expanding the range of genotypes amenable to transformation, thus, serving research and breeding initiatives to improve this important grass crop for forage and recreation.

Keywords: perennial ryegrass (*Lolium perenne* L.); *Agrobacterium*-mediated transformation; genome editing; functional genomics; doubled haploid (DH); tissue culture

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Citation: Grogg, D.; Rohner, M.; Yates, S.; Manzanares, C.; Bull, S.E.; Dalton, S.; Bosch, M.; Studer, B.; Broggini, G.A.L. Callus Induction from Diverse Explants and Genotypes Enables Robust Transformation of Perennial Ryegrass (*Lolium perenne* L.). *Plants* **2022**, *11*, 2054. https://doi.org/10.3390/plants11152054

Academic Editor: Iyyakkannu Sivanesan

Received: 31 January 2022
Accepted: 27 July 2022
Published: 5 August 2022

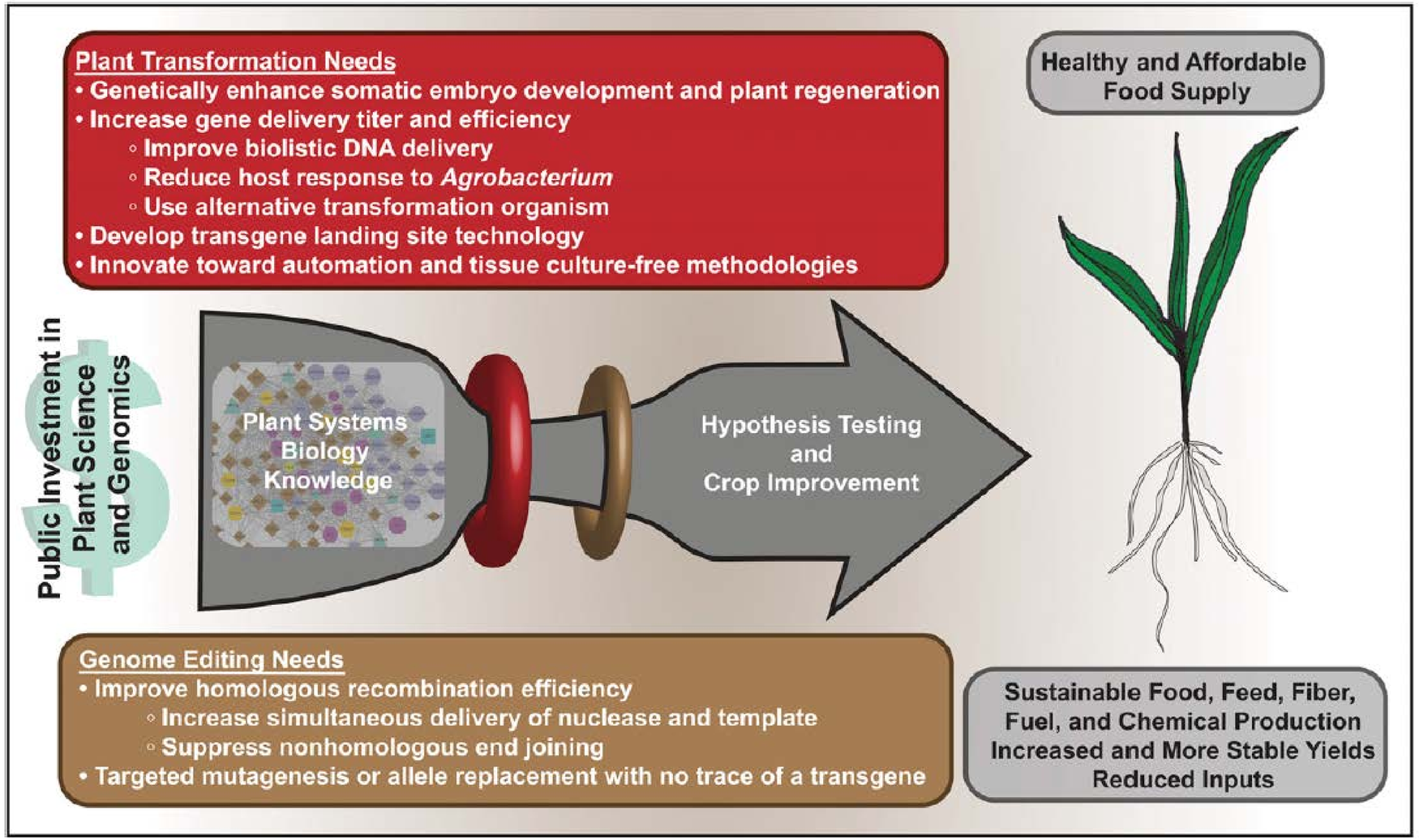
Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Perennial ryegrass (*Lolium perenne* L.) is an important grass grown in temperate regions and is used for cattle grazing, feeding, and recreation (gardens, parks and golf courses, for example) [1]. Despite the agronomic and economic importance of perennial ryegrass, the genetic gain for fundamental traits, such as yield, lags behind that of other major crops like wheat, maize, and soybean [2,3]. The genetic gains in perennial ryegrass are low because of many factors. For example, the establishment of genomic resources for perennial ryegrass is still in its infancy, limiting the exploitation of genomics-based breeding approaches. Additionally, perennial ryegrass is an outbreeding species because of a genetically determined self-incompatibility system [4], which limits the use of more effective breeding strategies [2].

Nevertheless, genomic resources are increasingly becoming available in perennial ryegrass and closely related species [5–7]. Access to high-quality genome assemblies has many benefits; for instance, they are essential for genome-wide association studies, can simplify map-based cloning and also help discover candidate genes [8]. In short, they make research faster and easier. While genome assemblies are helpful, their gene models are mostly predictions, so in vivo gene function characterization and verification are needed.

Bottlenecks in genome editing



Altpeter et al. (2016) Advancing crop transformation in the era of genome editing. The Plant Cell 28:1510-1520

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).

WPs

- 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

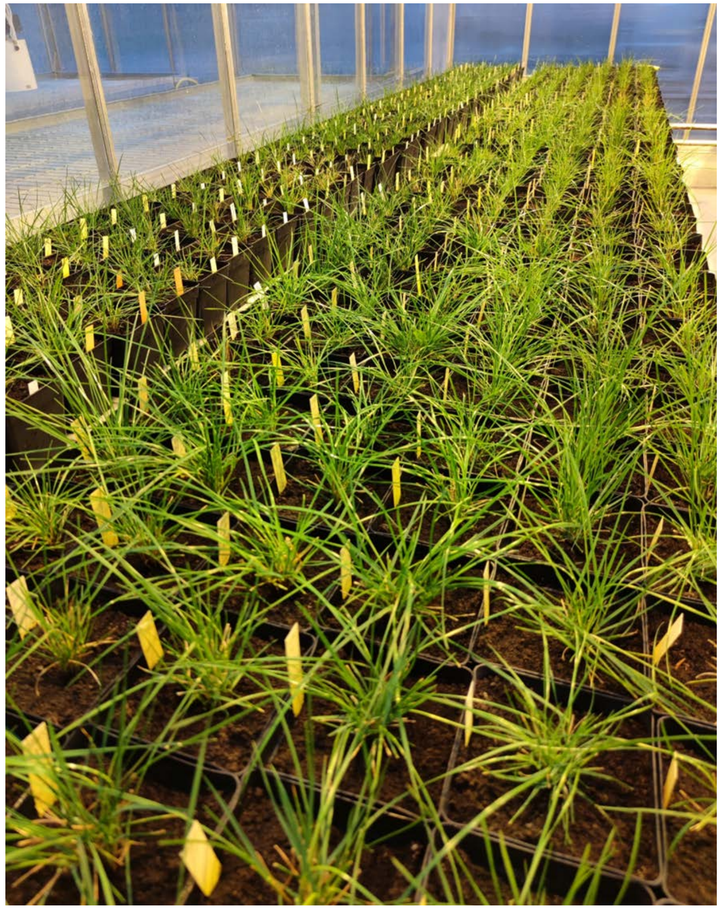
- WP3. Functional characterization of frost and drought candidate genes in perennial ryegrass by CRISPR-Cas9. Coordinator: TalTech; Involved partners: LU, NMBU

- 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

Deliverables

- Publications
    - 4 papers and 1 book chapter (open access)
- Scientific achievements
    - association mapping panel,
    - transcriptome study of drought and freezing
    - 10 gene edited plants assessed for drought or freezing tolerance,
    - 4 PhD students
- Joint application for Horizon Europe funding



Acknowledgements

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QUESTIONS?

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