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Transcriptomic Insights and Testing of Genome Editing Tools for Climate-Resilient Perennial Ryegrass

Ferenz Josef Sustek Sánchez

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Ferenz Josef Sustek Sánchez

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Transkriptoomi põhised teadmised ja genoomi täppismuutmise tööriistade testimine karjamaa raiheina kliimakindluse parandamiseks

FERENZ JOSEF SUSTEK SÁNCHEZ

TAL TECH
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List of publications

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I **Sustek-Sánchez F*,** Pashapu AR*, Kemešytė V, Kovi MR, Rognli OA, Sarmiento C, Rostoks N, Jaškūnė K **Growth under mild drought: transcriptomics comparison of perennial ryegrass genotypes with differential tolerance to water deficit** (manuscript submitted)

II Pashapu AR*, Statkevičiūtė G*, **Sustek-Sánchez F**, Kovi MR, Rognli OA, Sarmiento C, Rostoks N, and Jaškūnė K (2024) **Transcriptome profiling reveals insight into the cold response of perennial ryegrass genotypes with contrasting freezing tolerance**. Plant Stress 14:100598. https://doi.org/10.1016/j.stress.2024.100598

III **Sustek-Sánchez F**, Rognli OA, Rostoks N, Sõmera M, Jaškūnė K, Kovi MR, Statkevičiūtė G, and Sarmiento C (2023) **Improving abiotic stress tolerance of forage grasses – prospects of using genome editing**. Front Plant Sci 14. https://doi.org/10.3389/fpls.2023.1127532

IV **Sustek-Sánchez F,** Eelmets E, Nigul L, Kärblane K, Laasmaa M, Balode-Sausina M, Berzina SA, Ducis D, Kaktina E, Jaškūne K, Rognli OA, Rostoks N, and Sarmiento C (2026) **Isolation and transformation of perennial ryegrass (*Lolium perenne* L.) protoplasts for the *in vivo* assessment of guide RNAs editing efficiency**. Front Plant Sci 16. https://doi.org/10.3389/fpls.2025.1744085

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Author's contribution to the publications

Contribution to the papers in this thesis are:

I The author conducted part of the experiments (physiological measurements), analysed part of the data, generated graphics, and participated on writing, reviewing and editing of the manuscript.

II The author conducted part of the experiments (RNA extraction), analysed part of the data, and participated on writing, reviewing and editing of the manuscript.

III The author participated in the conceptualization of the article, drafted the manuscript, and participated on reviewing and editing the manuscript.

IV The author designed the study, conducted most of the experiments (protoplasts isolation, transformation and genome editing evaluation), analysed the resulting data, generated graphics, and wrote the manuscript.

Introduction

Currently, one of the main food sources for animal livestock are forage grasses. In Europe, *Lolium pyrene* L. (perennial ryegrass) is the most widely distributed and grown grass for this purpose. Perennial ryegrass is favoured for its high nutritional value, rapid regrowth, and tolerance to frequent cutting (McDonagh *et al.*, 2016). Unfortunately, *L. perenne*, a cool-season grass, struggles with low temperatures and water scarcity. This limits its potential to expand into northeastern European regions and adapt to emerging climate conditions driven by climate change, which feature more extreme temperatures and variable precipitation patterns (Buttler *et al.*, 2019; He and Li, 2020; Liu, Able and Able, 2022). Perennial ryegrass is adapted to temperate regions with mild winters and common precipitation (Ergon, 2017; Loka *et al.*, 2019), which makes it particularly vulnerable to increasingly frequent and severe droughts and cold episodes (He, He and Ding, 2018; IPCC, 2023). Drought stress commonly produces a decrease in water potential, stomatal closure, reduced photosynthetic activity and oxidative stress (Huang, DaCosta and Jiang, 2014; Lechowicz *et al.*, 2020). Low-temperature stress generally translates into reduced membrane fluidity, electrolyte leakage and activation of the ICE-CBF-COR signalling pathway (Thomashow, 1999; Rapacz *et al.*, 2014). The ICE-CBF-COR pathway is a central cold-response mechanism in which the ICE transcription factors induce the expression of the CBF genes, which in turn activate COR target genes responsible for changes in the metabolism and protection of cellular structures aiming to improve tolerance towards freezing (Thomashow, 1999). To maintain forage yield under these conditions, plants must be bred to cope effectively with stress. This underscores the need for a deeper understanding of the physiological and molecular bases of stress tolerance in *L. perenne*, enabling the selection of genotypes with superior tolerance mechanisms or the introduction of adaptive traits through genome editing.

Perennial ryegrass is an obligate outcrossing species, presenting a self-incompatibility mechanism. This reproductive trait results in a highly heterozygous genome that confers adaptability to changing environments but hinders genetic studies (Islam *et al.*, 2014; Pembleton *et al.*, 2015). While recent advances in genomic sequencing provide a promising basis for genetic studies in *L. perenne*, genotype-specific annotation remains a bottleneck in trait identification (Byrne *et al.*, 2015; Frei *et al.*, 2021; Nagy *et al.*, 2022). Nonetheless, transcriptomics can identify stress-responsive networks and transcripts. It has revealed conserved ABA-dependent signalling, ROS-scavenging enzymes, and osmolyte biosynthesis as core responses to drought and cold across diverse plant species, including major grass crops such as wheat (Wang *et al.*, 2020; Chen *et al.*, 2025). The ABA-dependent signalling is a central integrator of abiotic stresses (including drought, salinity and temperature related conditions), which acts by promoting responses like stomatal dynamics, gene expression regulation and metabolic reprogramming to cope with certain stressors (Cutler *et al.*, 2010). ROS-scavenging enzymes play a crucial role in response to abiotic stress by detoxifying excess of reactive oxygen species (ROS) resulting from stressful conditions, limiting damage towards lipids, proteins and DNA, while maintaining appropriate levels of ROS responsible for stress signalling processes (Foyer and Noctor, 2005).

One specific application of transcriptomics is identifying differentially expressed genes (DEGs) between plants showing varying responses to a particular stress. These DEGs can serve as candidate genes for genome editing to enhance plant tolerance to that stress. Genome editing, particularly CRISPR-Cas9, offers a precise route to modify candidate

genes. In monocots, *Agrobacterium*-mediated transformation of callus remains the standard delivery system of CRISPR-Cas encoding editing agents, yet regeneration efficiency is highly genotype-dependent (Chen *et al.*, 2022). Protoplast transformation enables evaluation of *in vivo* editing agents prior to their use in callus transformation, thereby accelerating the generation of edited plants by selecting agents with proven efficiency (Lin *et al.*, 2018; Fierlej *et al.*, 2022).

In this thesis, we applied transcriptomic analysis to study drought and low temperature stress (separately) in perennial ryegrass. We compared stress-sensitive and tolerant genotypes, allowing us to identify DEGs with dissimilar expression profiles between the genotypes. This revealed that specific pathways and regulatory networks were differentially modulated in the studied genotypes, providing insight into the higher susceptibility of the sensitive genotypes. The transcriptomic analysis was accompanied by physiological measurements that identified specific responses between the genotypes, such as electrolyte leakage resulting from freezing temperatures, or reduced leaf growth and relative water content due to drought stress. Moreover, a platform based on perennial ryegrass protoplasts derived from *in vitro* cultured tillers was generated to evaluate *in vivo* the editing efficiency of genome editing vectors targeting candidate genes capable of enhancing stress tolerance towards drought and low temperature stress in *L. perenne*.

By linking transcriptomics with a robust genome-editing testing platform, this work aimed to deliver a methodology and regulatory insights that can be used in breeding programmes to secure ryegrass productivity in a rapidly changing climate.

Abbreviations

ABA	Abscisic acid
BA/BAP	6-benzylaminopurine
BE	Base editing
CBF	C-repeat Binding Factor
CK	Cytokinin
COR	Cold-regulated genes/proteins
DEG	Differentially expressed gene
DSB	Double-stranded break
EGFP	Enhanced green fluorescent protein
Fv/Fm	Maximum quantum efficiency of Photosystem II
GE	Genome editing
GFP	Green fluorescent protein
GO	Gene ontology
GOI	Gene of interest
gRNA	guide RNA
HDR	Homology-directed repair
IAA	Indole-3-acetic acid
ICE	Inducer of CBF expression
indel	Insertion or deletion
KEGG	Kyoto encyclopedia of genes and genomes
LEA	Late embryogenesis abundant proteins
LER	Leaf elongation ratio
MAPK	Mitogen-activated protein kinase
NGS	Next generation sequencing
NGT	New genomic techniques
NHEJ	Non-homologous end joining
PAM	Protospacer adjacent motif
PE	Prime editor
PEG	Polyethylene glycol
PGR	Plant growth regulator
QTL	Quantitative trait loci
RNA-seq	High-throughput RNA sequencing
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RWC	Relative water content

SDN	Site-directed nuclease
SNP	Single nucleotide polymorphism
T-DNA	Transfer DNA
TF	Transcription factor
TIDE	Tracking of indels by decomposition
ZsGreen	Green fluorescent protein from Zoanthus sp.

1 Literature review

The following sections provide an overview regarding the main traits of the plant studied in this thesis, *Lolium perenne* L., about how two specific abiotic stresses affect grasses, and how new genomic techniques, in conjunction with the application of tissue culture methods, can be used to improve the tolerance of these plants towards the investigated stresses.

1.1 Perennial ryegrass

Lolium perenne, commonly known as perennial ryegrass, is among the most cultivated forage grasses in temperate regions, including Europe, where it is found throughout the continent (Sampoux *et al.*, 2013; Blackmore *et al.*, 2016; Zhu *et al.*, 2025). As a forage crop, the yield of *L. perenne* is determined by the biomass accumulation resulting from leaf growth (Shinozuka *et al.*, 2012; Gilliland, Ball and Hennessy, 2021). Some properties that make perennial ryegrass a highly valuable crop include its rapid establishment and grazing tolerance, together with excellent forage quality. These characteristics of *L. perenne* make it an important feedstock source (Young, Hume and McCulley, 2013; Zhu *et al.*, 2025). However, these valuable traits are strongly reduced or suppressed when *L. perenne* faces low temperatures or drought conditions. As a result, climate change is predicted to reduce the forage yield and quality of this plant significantly (Pasquali and Barcaccia, 2020; Miao *et al.*, 2022).

L. perenne is a diploid (2n = 14) monocot plant species that belongs to the Poaceae family. This family, commonly referred to as grasses, includes a wide variety of plants, such as widely cultivated cereals like barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (Shinozuka *et al.*, 2012). Grasses can be classified based on their optimal growth period into warm-season and cool-season grasses. Perennial ryegrass belongs to the latter group, since it presents higher biomass accumulation—which relates to growth—during spring and fall, when temperatures tend to be cooler and close to the range in which *L. perenne* grows best (the optimal growth temperature being 20–25 °C) (Wingler and Hennessy, 2016; Miao *et al.*, 2022). During summer and winter, perennial ryegrass experiences a dormancy or growth arrest stage that resumes in spring and fall. *L. perenne* can reproduce vegetatively through tillers and sexually, by flowering and seeds (Wingler and Hennessy, 2016; Pasquali and Barcaccia, 2020; Miao *et al.*, 2022). Flowering tends to happen in spring due to perennial ryegrass requirement of a vernalization period (which translates into days with short hours of daylight and low temperature) that happens during wintertime. Thus, the return to long photoperiods in spring promotes flowering (Shinozuka *et al.*, 2012; Pasquali and Barcaccia, 2020). Perennial ryegrass has a wind-pollinated sexual reproduction strategy which presents self-incompatibility, making this plant an obligate outcrossing species with a highly heterogenous genome (Shinozuka *et al.*, 2012; Pembleton *et al.*, 2015; Zhu *et al.*, 2025). While this mechanism provides the plants with a healthy gene pool that aims to preserve its adaptability and resilience to environmental stress (Blackmore *et al.*, 2016), it also proves challenging when performing genetic studies (Shinozuka *et al.*, 2012; Islam *et al.*, 2014; Pembleton *et al.*, 2015). Until recently, no whole genome sequence was available for *Lolium perenne*. At the beginning of this decade, two independent chromosome-level assemblies were published, each corresponding to a different perennial ryegrass genotype (Byrne *et al.*, 2015; Frei *et al.*, 2021; Nagy *et al.*, 2022). In both cases, the annotation of the genome is only partially complete and requires manual curation when

trying to identify regions of interest such as start codons. Due to the high heterozygosity of this plant, the genomic background tends to be specific of particular cultivars. This creates a bottleneck when studying the genetic basis of phenotypes of interest such as drought or low temperature tolerance (Pembleton *et al.*, 2015; Blackmore *et al.*, 2016; Pasquali and Barcaccia, 2020; Miao *et al.*, 2022). Tissue culture, in conjunction with genome editing techniques, holds the potential to overcome these constraints. Combined with transcriptomics analysis of cultivars exhibiting relevant phenotypes, these methods can be employed to breed new varieties with traits that can help plants overcome the challenges posed by the ever-changing climate and the increased demand for food supplies.

1.2 Abiotic stress in grasses

Climate change is leading to extreme shifts in temperature and irregular rainfall patterns, among other variations (IPCC, 2023). These environmental fluctuations significantly affect agriculture, since plants are sessile organisms that cannot escape adverse conditions. Abiotic stresses result mainly from the physical and chemical conditions of the environment (He, He and Ding, 2018). Plants respond to these environmental changes by altering their metabolism, physiology, and morphology (Sustek-Sánchez *et al.*, 2023). Drought and extreme temperatures are frequent stressors for plants, with their impact exacerbated by climate change, directly affecting growth, fertility, and survival.

Grasses are fundamental to the wellbeing of ecological systems and global food security. As they cover over 70% of the world's cultivated land, understanding how these plants respond to abiotic stresses is crucial (Loka *et al.*, 2019). Cool-season grasses, including perennial ryegrass, are highly susceptible to the aforementioned climatic variations, since they have evolved and adapted to regions where these stressors are not as common, persistent and intense as they are expected to be (Ergon, 2017; Loka *et al.*, 2019; Schubert *et al.*, 2019). Perennial grasses, in particular, must be able to cope and adapt to the abiotic stresses that they are continuously exposed to, underlining how impactful these can be in terms of maintaining desired growth and yield (Zwicke *et al.*, 2015; Loka *et al.*, 2019; Miao *et al.*, 2022).

An interesting aspect of abiotic stress in plants is the concept of stress resistance and tolerance. While from a physiological point of view stress tolerance can be considered as the ability of a particular plant to survive under specific stress conditions, it is a common consensus to consider that tolerance is part of a plant's ability to resist the consequences of a stress (Levitt, 1980a, 1980b). Therefore, if a plant can maintain stable growth and thus biomass accumulation and yield, this organism is referred to as resistant to said stress (Ergon, 2017; Bandurska, 2022). In this sense, resistance is normally defined by the ability of a plant to use a combination of avoidance and tolerance mechanisms, and is commonly the result of physiological, biochemical and precise gene regulation processes (Levitt, 1980a, 1980b). Additionally, two other concepts are closely related to stress resistance and tolerance: adaptation and acclimation. On one hand, adaptation is an evolutionary process that happens in a population level, across many generations, that is based on heritable traits that have been enriched or fixed through natural selection or selective breeding. On the other hand, acclimation (also known as hardening or plastic adjustment) occurs in an individual scale through the lifespan of a plant; it is based on preexisting traits and on immediate and reversible responses (Yamaguchi-Shinozaki and Shinozaki, 2006). An example of adaptation can be found in plants with inherently deeper root systems, which confer them with higher tolerance to drought stress (Kim *et al.*,

2024), while acclimation is the reason behind plants ability to close their stomata under water deficit conditions (Yamaguchi-Shinozaki and Shinozaki, 2006).

Understanding the mechanisms that underpin grasses' ability to perceive and respond to environmental stresses to maintain their growth and reproductive potential is vital for sustaining highly productive agriculture in the face of unfavourable climatic conditions. The following subsections cover general aspects of two abiotic stresses that can severely hinder grasses growth and survival, directly impacting crop yield and food availability.

1.2.1 Drought stress

The projected changes in climate are expected to lead to higher temperatures and altered precipitation patterns, which will translate in more common and severe drought periods. Drought is a significant environmental factor that limits crop yield. Non-tolerant plants tend to decrease biomass accumulation, while drought tolerant ones can maintain growth under temporary and non-extreme water deficit, preserving biomass accumulation (Jaškūnė *et al.*, 2020). Thus, tolerance to drought is a crucial trait for ensuring the stability of food and feed sources in the new climatic conditions.

Although there is no clear agreement on the exact definition of drought, it is widely recognized as a result of extended periods of insufficient precipitations, leading to water shortages that negatively impact ecosystems, agriculture, and human activities (Wilhite and Glantz, 1985). From a physiological perspective, drought is the alteration of normal plant functions due to reductions in water potential and turgor (Hsiao, 1973; Loka *et al.*, 2019). As is the case with many other abiotic factors, plants can cope with drought in different ways depending on their native growing regions and climates. While in temperate regions drought is not so common, and is generally restricted to short periods during summer, plants in arid regions have developed unique adaptations to survive prolonged dry periods, including morphological, physiological, and specific gene regulation mechanisms (Loka *et al.*, 2019; Kirschner, Xiao and Blilou, 2021). For example, some plants have thicker leaf cuticles with higher wax content that help them reduce water loss from evapotranspiration (Shepherd and Wynne Griffiths, 2006). Other plants exhibit finely tuned stress response systems that enable them to manage the accumulation of oxidative molecules caused by drought (Laxa *et al.*, 2019).

Plants with different life cycles—i.e., annual or perennial plants—employ distinct strategies to cope with reduced water availability (Friedman, 2020). Perennial plants, such as *L. perenne*, respond to drought conditions through mechanisms of avoidance and tolerance. In contrast, annual plants predominantly evade drought stress by completing their reproductive cycle early, thereby ensuring adequate seed production (Friedman, 2020; Keep *et al.*, 2021). Drought avoidance aims to either maintain high water potential or to prevent it from decreasing too much. Grasses can achieve this by using several mechanisms that either aim to conserve optimal levels of water intake or to reduce water loss (Malinowski and Belesky, 2000; Zwicke *et al.*, 2015). By altering their root system, for example by growing larger roots or stimulating the development of lateral ones, grasses can control their ability to uptake water (Loka *et al.*, 2019). To avoid severe water loss, plants can alter the morphology of their leaves through stomatal modifications, including closing part of them or reducing their number in newly formed leaves (Huang, DaCosta and Jiang, 2014; Loka *et al.*, 2019). Tolerance on the other hand, is characterized by molecular changes such as the accumulation of different carbohydrates and secondary metabolites with the goal of preserving osmotic potential and preventing the

accumulation and consequent damage from oxidative molecules like reactive oxygen species (ROS) (Zwicke *et al.*, 2015; Lechowicz *et al.*, 2020).

Plants can undergo a different array of physiological, molecular and morphological changes in response to drought stress. Some of the main physiological responses in plants are related to the water content in cells and tissues, which is regulated by stomatal conductance. In turn, this stomatal regulation directly impacts gas exchange, and therefore the ability of plants to fix carbon through photosynthesis (Malinowski and Belesky, 2000; Loka *et al.*, 2019). Under drought conditions, the relative water content (RWC) of cells is reduced, to which grasses tend to respond by closing their stomata to preserve their water content. A key component of this stomatal regulation is the phytohormone abscisic acid (ABA), whose levels increase in response to drought leading to stomatal closure (Loka *et al.*, 2019; Lechowicz *et al.*, 2020). However, drought tolerant plants tend to have reduced water loss under drought conditions, and when they do, they can cope with reduced RWC by accumulating osmolytes, such as proline, that allow them to preserve their osmotic balance (Miao *et al.*, 2022). Stomatal closure also leads to reduced photosynthetic activity that can translate in decreased growth levels. Photosynthesis is also affected under drought conditions by non-stomatal related reasons, like reduced photochemical efficiency in result of lower water abundance (Huang, DaCosta and Jiang, 2014; Lechowicz *et al.*, 2020). Water deficit also induces oxidative stress in plants, which can be counteracted by the synthesis of secondary metabolites and specialized enzymes such as dismutases and catalases. These enzymes can protect cells and cellular components from oxidative damage by scavenging ROS (Loka *et al.*, 2019; Lechowicz *et al.*, 2020; Miao *et al.*, 2022). The previously mentioned ABA is a key regulator of drought responses in plants. Besides its involvement in regulating stomatal closure, ABA is also responsible for inducing the ABA-dependent signalling pathways, which are one of the principal molecular responses towards drought (Mehrotra *et al.*, 2014; Lechowicz *et al.*, 2020; Miao *et al.*, 2022). ABA induces expression of several transcription factors (TFs), like those belonging to the NAC, MYB and WRKY families, which are responsible for the downstream regulation of stress responsive genes. Plants with higher expression of some of these TFs have been shown to present enhanced drought tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007; Lechowicz *et al.*, 2020; Miao *et al.*, 2022). Similarly, the pathways involved in drought response are also tightly regulated by calcium ions and ROS, which can activate signal transducers such as SnRK2s kinases (sucrose non-fermenting 1-related protein kinases) (Zhu, 2016; Lechowicz *et al.*, 2020; Miao *et al.*, 2022). An interesting morphological response to drought stress in grasses can be observed in their leaves. Old leaves can undergo senescence, i.e., programmed cell death, to preserve the water content of the plants and to enhance the survival of meristematic tissues located in the basal or crown region of the grass (Munné-Bosch and Alegre, 2004; Loka *et al.*, 2019; Miao *et al.*, 2022).

Knowledge regarding how plants respond to drought stress is continuously evolving and expanding, thanks to the advance of novel techniques such as transcriptomics. Further comprehension of these mechanisms can be key to develop new grasses varieties through genome editing methods, which could speed up the natural adaptation of plants to the new climatic conditions.

1.2.2 Low temperature stress

One significant impact of climate change is the modification of temperature conditions, which directly influences and restricts plant growth and development. Consequently, it greatly affects crop yield, which has direct implications for food security (FAO, 2019). Temperature variations are believed to cause two types of abiotic stress: heat stress and cold stress (Sustek-Sánchez *et al.*, 2023). In plants, cold or low temperature stress can be divided according to the temperature level in chilling and freezing stress. Chilling stress comprises exposure to low temperatures above the freezing point, i.e., above 0 °C but below 15 °C. While freezing stress, as the name suggests, is the result of sub-zero temperatures, which tend to be accompanied by cell dehydration and the formation of extracellular ice (Thomashow, 1999; Rapacz *et al.*, 2014; Ergon, 2017). Like is the case with drought stress, there are different strategies to cope with low temperature stress depending on the life cycle of plants. Similar to their drought response, annual plants employ avoidance mechanisms, typically completing their reproductive cycle before the onset of winter. Perennial plants, conversely, utilize various strategies to mitigate the damage caused by cold stress, aiming to survive winter conditions (Thomashow, 1999; Rapacz *et al.*, 2014; Ergon, 2017).

Cool-season grasses, including *L. perenne*, are adapted to temperate climates where stress caused by low temperatures can hinder growth and productivity (Ergon, 2017). During autumn, when temperatures decrease and photoperiods shorten, cool-season grasses can experience a reversible process, known as cold acclimation, that prepares plants for sub-zero temperatures and enhances their chances of surviving winter conditions (Thomashow, 1999). During cold acclimation, plants undergo a series of physiological, molecular and biochemical modifications, that allow the crown-region of grasses (where a great portion of the meristems are located) to sustain very low temperatures on the range of -15 °C. Non-acclimated plants, on the other hand, have a high chance of not surviving these kinds of sub-zero temperatures (Thomashow, 1999; Kovi, Ergon and Rognli, 2016; Ergon, 2017). Growth cessation can occur in conjunction with cold acclimation or even precede it, enabling plants to divert energy and resources towards preservation processes rather than biomass accumulation (Kovi, Ergon and Rognli, 2016; Ergon, 2017). Some of the adjustments grasses go through during cold acclimation include physiological changes such as the accumulation of osmoprotectants like fructans, changes in the lipidic composition of cellular membranes, and the synthesis of anti-freeze proteins. These modifications allow plants to prevent the formation of ice crystals and to counteract the effects of oxidative stress and dehydration (Thomashow, 1999; Rapacz *et al.*, 2014; Ergon, 2017). The ability to cope with dehydration caused by sub-zero temperatures is highly linked to freezing tolerance (Rapacz *et al.*, 2014; Ergon, 2017). For example, perennial ryegrass plants capable of accumulating more fructans after cold acclimation can maintain higher RWC in freezing conditions than the plants that do not (Hisano *et al.*, 2004; Abeynayake, Etzerodt, *et al.*, 2015).

A process commonly related with cold acclimation is vernalization, which is a mechanism that plants use to acquire floral competence, i.e., the ability to produce inflorescences (Prášil, Prášilová and Pánková, 2004; Dhillon *et al.*, 2010). Vernalization also requires low temperature conditions and short photoperiods, common during autumn months, which explains the connection between both processes (Winfield *et al.*, 2010; Ergon, 2017). Plants requiring vernalization periods to flower, tend to have prolonged vegetative growth seasons, which in turn provides plants with prolonged time to undergo cold acclimation. Therefore, while there is not a direct genetic link between cold tolerance

and vernalization, plants that must go through a vernalization period tend to have better survival rates under low temperature stress (Prášil, Prášilová and Pánková, 2004; Dhillon *et al.*, 2010; Winfield *et al.*, 2010).

After winter, when temperatures start to rise above 10 °C and photoperiods lengthen, grasses can undergo deacclimation, which lowers the freezing tolerance of plants and triggers remobilization of resources with the aim of activating plant growth during spring (Rapacz *et al.*, 2014; Dalmannsdottir *et al.*, 2017; Ergon, 2017). This process can be counterproductive in some cases, since increased temperature during winter can also trigger deacclimation. If this happens, plants reduce their reserves of osmoprotectants and downregulate important stress related genes, such as the *COR* genes, leading to a decrease in the plants' ability to cope with freezing temperatures and increasing the chances of dehydration stress and death (Rapacz *et al.*, 2014; Kovi, Ergon and Rognli, 2016; Ergon, 2017).

One of the first impacts of low temperature stress is the decrease in cellular membranes' fluidity. Temperatures below 10 °C change the physical properties of the lipids present in cellular membranes, leading to increased viscosity and rigidification. This decrease in fluidity translates in reduced diffusion of proteins and lipids, and in leakage of metabolites and ions (or electrolytes) (Thomashow, 1999; Kurepin *et al.*, 2013). If temperatures decrease below zero, extracellular ice can form, leading to fractures in the already rigid membranes that can derive in water leakage and thus dehydration stress. To counteract this rigidification, plants can modify the fatty acid composition of their cellular membranes, preserving membrane fluidity by increasing the ratio of unsaturated lipids (Thomashow, 1999; Kurepin *et al.*, 2013; Rapacz *et al.*, 2014; Ergon, 2017). Osmoprotectants or osmolytes, like proline, can also be present and redirected into cellular membranes, to create an interphase that contributes to preserve membrane fluidity. Additionally, proteins such as dehydrins and other members of the LEA family, can also bind to cellular membranes to prevent tight packing of lipids, and therefore reducing the temperature at which membranes will become more viscous (Thomashow, 1999; Kurepin *et al.*, 2013; Rapacz *et al.*, 2014). Electrolyte leakage can be measured to evaluate the ability of grasses to sustain periods of low temperature conditions, since leakage is directly related to cellular membrane damage. Therefore, plants that present reduced electrolyte leakage under similar conditions can be considered as tolerant to those temperatures (Kurepin *et al.*, 2013; Ergon, 2017). This is evident in *L. perenne*, where cold-sensitive genotypes, after cold acclimation, exhibit greater electrolyte leakage when exposed to low temperatures compared to tolerant genotypes (Abeynayake, Etzerodt, *et al.*, 2015).

Drought and cold stress activate similar responses in grasses and share similar regulatory networks and pathways (Thomashow, 1999; Kurepin *et al.*, 2013; Caccialupi *et al.*, 2023). One such case is the accumulation of osmolytes and other molecules, like soluble carbohydrates, to protect cells and cellular components from low temperature induced damages. Some of these molecules, including fructans and sucrose, allow plants to reduce the freezing point of their components and to prevent oxidative stress (Rapacz *et al.*, 2014; Ergon, 2017). Another important response of plants when temperatures decrease, is the accumulation of amino acids such as proline, which helps to preserve the stability of membranes and scavenges ROS molecules. Additionally, proline has been shown to act as a source of energy and nitrogen after deacclimation (Thomashow, 1999; Szabados and Savouré, 2010; Kurepin *et al.*, 2013; Rapacz *et al.*, 2014; Ergon, 2017). Like in the case of drought, low temperature stress can also lead to the accumulation of ROS

molecules by disrupting optimal electron transport in mitochondria and chloroplasts. To stop this accumulation from damaging cells, plants can increase the synthesis of antioxidant enzymes by upregulating the genes encoding said proteins under stressful conditions (Thomashow, 1999; Kurepin *et al.*, 2013; Rapacz *et al.*, 2014).

The phytohormone ABA also plays an important role in low temperature stress, by acting as an initial signalling agent. In this sense, ABA activates ABA responsive elements and transcription factors that act in conjunction with the main cold induced signalling pathway, the ICE-CBF-COR pathway, to upregulate the synthesis of specific metabolites and proteins like dehydrins (Thomashow, 1999; Kurepin *et al.*, 2013; Kovi, Ergon and Rognli, 2016). Furthermore, ABA also suppresses plant growth by antagonizing other plant hormones, such as gibberellins, diverting energy and carbon resources towards cold tolerance mechanisms (Kurepin *et al.*, 2013; Kovi, Ergon and Rognli, 2016; Ergon, 2017).

As previously mentioned, a key element of the molecular response of grasses towards cold stress is the activation of the ICE-CBF-COR pathway. ICE (Inducer of CBF Expression) proteins are TFs whose expression is induced when low temperatures generate mechanical changes in cellular membranes. These ICE proteins in turn bind to the *CBF* (C-repeat Binding Factor) genes to promote their expression, integrating cold stress signals and gene modulation. The CBF proteins bind and induce the transcription of *COR* (Cold-Regulated) genes, amplifying the downstream regulation of cold response mechanisms (Thomashow, 1999; Kurepin *et al.*, 2013; Kovi, Ergon and Rognli, 2016; Caccialupi *et al.*, 2023). This family of more than 100 genes encode proteins in charge of several responses towards cold stress, including dehydrins, antioxidant enzymes and osmolyte synthesis (Thomashow, 1999; Kurepin *et al.*, 2013; Kovi, Ergon and Rognli, 2016).

Similar to its importance in drought stress, the crown region plays a crucial role in a plant's ability to cope with and survive cold stress (Rapacz *et al.*, 2014; Kovi, Ergon and Rognli, 2016; Ergon, 2017). This region represents a key regulator of the ICE-CBF-COR signalling, where all the different mechanisms responsible for low temperature stress converge. For example, rehydration of the crown tissue can induce deacclimation and thus the downregulation of CBFs and their associated processes. If the crown region of a grass survives winter, this plant presents a higher chance of resuming normal growth and biomass accumulation (Kurepin *et al.*, 2013; Rapacz *et al.*, 2014; Kovi, Ergon and Rognli, 2016; Ergon, 2017).

Cold acclimation is expected to be highly impacted by climate change. The new climatic conditions will bring longer autumn periods, which will delay or totally suppress the ability of plants to acclimate to the lower temperatures from winter periods. Additionally, premature deacclimation processes can also become more usual due to winters becoming milder, hence presenting higher temperatures than usual (Rapacz *et al.*, 2014; Kovi, Ergon and Rognli, 2016; Ergon, 2017). In concordance, snow coverage will be reduced by higher temperatures, which in turn increases the risk of plants from suffering frost damage when temperatures go below zero (Rapacz *et al.*, 2014; Ergon, 2017).

Advancements on how plants respond to cold stress will provide crucial information that can help develop novel grass varieties using genome editing, thereby potentially preserving or even increasing yield and productivity.

1.2.3 Transcriptomics and its use in plant abiotic stress

As sessile organisms, plants are continuously exposed to changing conditions, including abiotic stress, which can significantly hinder their growth and productivity (Li *et al.*, 2017; Da Ros *et al.*, 2023). Therefore, understanding how plants respond to these unfavourable conditions is paramount for maintaining high yields and productivity, especially in fluctuating climatic environments exacerbated by climate change (Mahalingam *et al.*, 2022; Da Ros *et al.*, 2023). Transcriptomics is the field that studies and quantifies all RNA molecules produced from an organism's genome in a specific cell, tissue, or condition, using high-throughput technologies (e.g., RNA-seq, microarrays) to uncover patterns of gene activity, regulation, and functional pathways (Wang, Gerstein and Snyder, 2009). The ability of transcriptomics to capture dynamic changes in gene expression makes it highly suitable for studying the effects of stress and stress-derived responses in plants (Choudhury *et al.*, 2021). Through this, the regulatory networks plants utilize under specific conditions can be uncovered, providing valuable information that helps understand how plants cope with stress. This, in turn, enables the deciphering of factors that make an organism more susceptible or tolerant to unfavourable conditions (Waititu *et al.*, 2021; Chen *et al.*, 2025). Ultimately, this knowledge can be applied to generate new crop varieties with enhanced abilities to cope with stresses (Wei *et al.*, 2021; Chen *et al.*, 2025).

Transcriptomics studies have been extensively used to identify differentially expressed genes (DEGs) that participate in key regulatory pathways (Cohen and Leach, 2019; Choudhury *et al.*, 2021). These DEGs, which are transcripts showing varying expression levels among different plant varieties studied under similar conditions, can shed light on how these varieties cope with a given stress. This insight is highly valuable for identifying targets for genome editing, aiming to improve tolerance to specific stresses. For example, DEGs identified between drought sensitive and tolerant wheat and maize genotypes provide information about how different varieties withstand lack of watering (Waititu *et al.*, 2021; Xi *et al.*, 2023). In this sense, researchers identified a transcription factor in maize, *ZmbHLH124*, that increased the drought tolerance of plants when overexpressed (Wei *et al.*, 2021).

There are different methods used for transcriptomics analysis, including SAGE (serial analysis of gene expression), DNA microarrays, and RNA-seq (high-throughput RNA sequencing). This latter technique involves the qualitative and quantitative analysis of the coding and non-coding RNA molecules of a specific sample. With its higher sensitivity, deeper coverage, and lower costs, RNA-seq analysis can be performed even without a reference genome (by conducting de novo assemblies), making it the default method for transcriptomics analyses. The relevance of RNA-seq in stress studies is also related to this technique allowing for the detection of subtle changes of transcript abundance, which is key to uncover stress-induced transcriptome dynamics and responses. RNA-seq analysis generate huge data sets, that require intensive bioinformatics analysis to fully interpret their significance. Integrating the RNA-seq data generation with bioinformatic workflows like principal component analysis (PCA), differential expression analysis (such as DESeq2), gene ontology (GO) and pathway enrichments (KEGG), has enabled researchers to extract profound mechanistic insights from extensive datasets, elucidating stress tolerance strategies across both molecular and systems levels (Love, Huber and Anders, 2014; Wu *et al.*, 2021). For example, experiments in rice have shown that stressors in general upregulate important hormone-related genes, such as the ones involved in the ABA signalling pathway, while genes linked to photosynthesis are downregulated (Cohen and

Leach, 2019). In wheat, molecular markers used to identify and select stress-tolerant genotypes have been developed thanks to data derived from transcriptomics assays (Shah *et al.*, 2018). Furthermore, transcriptomics studies have provided a deeper understanding of how transcription factors play a central role in the regulation of abiotic stress responses. For instance, in rice, bZIP TFs seem to be involved in both biotic and abiotic responses, but more of these TFs appear to be upregulated by abiotic stress (Cohen and Leach, 2019). Moreover, thanks to the array of data collected by transcriptomics experiments, new insights have arisen into the regulatory mechanisms and pathways of plant stress responses, allowing to construct detailed gene regulatory networks. Evaluating the cold tress responses of maize seedlings with different stress tolerance, allowed to identify several key genes with high connectivity (hub genes) involved in low-temperature responses (Yu *et al.*, 2023). Similarly, studying the effect of early drought stages in wheat lead to the discovery of two possible master regulators of early drought response (Barratt *et al.*, 2023).

Transcriptomics studies can be integrated together with other omics research, such as metabolomics (focused on the metabolite profile under specific conditions) and proteomics (dedicated to studying protein expression patterns) to further assess plants responses towards stress. This multi-omics approach can provide a holistic and more realistic understanding of the mechanisms and pathways plants use to cope with various stressful conditions, effectively bridging genotypic and phenotypic variations. Hence, providing a better understanding of the physiological impacts of stress and allowing the identification of markers and candidate genes suitable for breeding and genome editing purposes (Pan *et al.*, 2022; Fu *et al.*, 2024). Coupling transcriptomics and metabolomics data has permitted to establish the relationship between the accumulation of secondary metabolites with the upregulation of antioxidant-linked transcripts, aiming to reduce the impact of oxidative damage resulting from drought stress in wheat leaves, roots, and seedlings (Fu *et al.*, 2024; Hu *et al.*, 2024; Li *et al.*, 2025). Also in wheat, multi-omics analysis has been applied to create an atlas of the different manners in which plants respond to different abiotic stresses, allowing the researchers to identify a possible master regulator, *TaWRKY33*, suitable for genome editing approaches aiming to enhance the tolerance to several stresses (Da Ros *et al.*, 2023).

In summary, transcriptomics analysis stands as a cornerstone of contemporary plant molecular biology, fundamentally enhancing our comprehension of abiotic stress responses. By comprehensively profiling gene expression changes induced by abiotic stressors, transcriptomics has elucidated crucial regulatory networks and physiological processes that underpin plant adaptation and survival. The integration of transcriptomics with other omics disciplines and advanced computational analyses is instrumental in identifying robust genetic targets for developing stress-resilient crop varieties. Continued innovation in transcriptomics and multi-omics integration holds significant promise for addressing global challenges related to food security and sustainable agriculture in the context of climate change.

1.3 Plant tissue culture

Plant tissue culture is an important advancement of plant biotechnology based on the totipotency of plant cells. This characteristic allows plant cells to differentiate into any organ or tissue, which can lead to the regeneration of a whole plant from an explant—a tissue or organ cultured in a nutrient rich media (Long *et al.*, 2022). Said totipotency is the basis of some applications of plant tissue culture, which can be used for clonal

propagation, contributing to genetic manipulation and germplasm conservation (Cruz-Cruz, González-Arnao and Engelmann, 2013). Plant tissue culture is a concept that englobes the controlled growth of different kinds of cells, tissues and organs. Depending on which part of the plant is grown *in vitro*, plant tissue culture can be classified in: cell culture (including protoplast and cell suspension culture), tissue culture (growing differentiated tissues and callus) and organ culture (growing roots or shoots, among other plant organs) (Loyola-Vargas and Ochoa-Alejo, 2018).

The emergence of plant tissue culture is considered to have begun in the early 18th century, when Austrian botanist Gottlieb Haberlandt attempted to cultivate fragments of leaves in culture media supplemented with sucrose. While he managed to keep them alive and increase their size, he could not induce their differentiation. Haberlandt hypothesized that plant fragments could be cultured in artificial media and that plant cells had the ability to regenerate into a whole plant, i.e., plant cells present totipotency (Haberlandt, 1902; Fehér, 2019). Later, in the 1930s and 40s, the ideas and hypothesis of Haberlandt were confirmed. Philip White managed to establish the permanent culture of tomato root tips in liquid media, demonstrating the potential for unlimited growth of cultured cells (White, 1934; Fehér, 2019). He later succeeded on generating and growing what he believed were close to undifferentiated cells from young tumour forming stems of hybrid Nicotiana plants (*N. glauca* X *N. langsdorffii*), which proliferated in clumps or masses of cells (White, 1939). In parallel, two French scientists managed to induce the growth of undifferentiated cells by culturing root caps of carrots, in a medium containing IAA (indole-3-acetic acid) (Gautheret, 1939; Nobécourt, 1939). Together, their works layered the foundations of callus culture (Ikeuchi, Sugimoto and Iwase, 2013).

A critical aspect of plant tissue culture is the use of an appropriate culture medium. Culture media supply essential macro and micronutrients, together with vitamins and carbohydrate sources (Sudheer *et al.*, 2022). Additionally, plant growth regulators (PGRs) or phytohormones can be used to control differentiation and development of specific organs or tissues, including the formation of callus or callogenesis (Sehgal and Joshi, 2022; Sudheer *et al.*, 2022). Different PGRs, or a combination of them, are used to guide the formation of different tissues. For example, auxins can induce root formation while cytokinins (CKs) promote shoot generation. Therefore, a specific ratio of these regulators is necessary to control the differentiation of explants (Long *et al.*, 2022). Folke Skoog and colleagues were the first to report how using different levels of PGRs could determine the differentiation of cultured cells and therefore the formation of different organs and structures. Skoog and colleagues observed that the auxin IAA inhibited the formation of shoots in cultures derived from tumour forming stems of tobacco plants, which White had previously stated that spontaneously produced shoots. Conversely, high levels of auxin in the medium induced the formation of roots in the cultured tissue. Furthermore, Skoog and colleagues used an adenine derivative known as kinetin (a phytohormone from the CKs family) to discover that adjusting the ratio of cytokinin and auxin present in the media enabled the differentiation of either shoots or roots and controlled the growth of undifferentiated cells or callus. Their discoveries established the basis of modern tissue culture and further confirmed the hypothesis of Haberlandt regarding the totipotency of plant cells (Skoog and Tsui, 1948; Skoog and Miller, 1957; Ikeuchi, Sugimoto and Iwase, 2013). Other parameters such as temperature, humidity, photoperiod and light intensity need to be closely regulated to provide the optimum conditions for explants to grow and differentiate *in vitro* (Espinosa-Leal, Puente-Garza and García-Lara, 2018). In summary, being able to fine tune the appropriate media compositions, including

specific ratios of PGRs, can be used to facilitate downstream applications of tissue culture such as large-scale micropropagation of commercially relevant plants. A significant challenge of tissue culture lies in the genotype-dependent nature of cultured cells responses. This often leads to the creation of species- or even cultivar-specific protocols to optimize shoot proliferation and callus induction. Strategies to address these limitations include preconditioning treatments, modifications to explant source and developmental stage, and the implementation of innovative elicitors and additives to enhance morphogenetic responses (A. Hussain *et al.*, 2012; Long *et al.*, 2022). The integration of advanced genome editing tools like CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas (CRISPR associated proteins) with tissue culture methodologies facilitates precise genetic modifications and accelerates the breeding of superior plant varieties that exhibit enhanced agronomic characteristics. (A. Hussain *et al.*, 2012; Park, 2021c; Gantait *et al.*, 2022). This section of the literature review focuses on two applications of tissue culture commonly used when working with cereals or grass species, and that were utilized through the experiments leading to this thesis: *in vitro* propagation and callus culture.

1.3.1 *In vitro* propagation

Among its advantages, plant tissue culture is a technology that allows to bypass some of the limitations of conventional propagation (sometimes referred to as macropropagation), since it is based on culturing different explants in controlled and aseptic, i.e., *in vitro*, conditions (A. Hussain *et al.*, 2012; Hasnain *et al.*, 2022). For example, micropropagation (also known as *in vitro* tillering in grass species) can be used to quickly and efficiently multiply uncontaminated or disease-free plant material (Park, 2021b; Krishna *et al.*, 2022). *In vitro* propagation or tillering relates to the induction of shoot multiplication, often from the meristems or axillary buds of a shoot, with the goal of producing clones of a parent plant under sterile conditions (Park, 2021c). This technique is currently used in breeding programs of important crops such as rice, and it is extensively used for the propagation of ornamental plants (Neumann, Kumar and Imani, 2020). Moreover, *in vitro* propagation can be used to overcome factors that limit conventional vegetative propagation, like low rooting capacity, specific growing seasons and weather conditions; and to avoid the spread of pathogens such as virus. In addition, this technique is especially useful when aiming to preserve individual plants with specific traits, such as resistance to a particular stress, or when working with plants with difficult genetic traits like sexual sterility or self-incompatibility (Neumann, Kumar and Imani, 2020; Krishna *et al.*, 2022).

The process of establishing an *in vitro* propagation culture starts with the selection of the appropriate initial material used for the explant or plantlet formation. In grasses, this tends to be a shoot meristem or meristematic region that has been previously sterilized to avoid spreading contaminations onto the newly developed explants (Shahzad *et al.*, 2017). Meristems are regions of a plant that contain a pool of undifferentiated cells that are involved in growth and development of organs and structures. For example, shoot apical meristems generate new leaves, stems and flowers, making them a suitable material for the *in vitro* multiplication of explants (Thompson, 2014). The “clean” meristems are placed in specific culture media, containing a mix of CKs and auxins that trigger the formation of multiple shoots, similarly to how plants produce new tillers. CKs play a crucial role on stimulating shoot multiplication. Specific CKs have different roles in shoot formation. BA/BAP (6-benzylaminopurine) is used in tillering to break apical

dominance and promote the proliferation of lateral shoots, which increases the rate in which tillers can be formed (Sehgal and Joshi, 2022). That said, it is important to mention that the induction of *in vitro* tillering is highly genotype dependent, and therefore the specific proportion of PGRs can have a deep impact on the speed at which tillers multiply (Loyola-Vargas and Ochoa-Alejo, 2018; Bidabadi and Jain, 2020). Once tillers are established and grown, they are placed in a medium containing a different mix of PGRs, normally with a higher proportion of auxins than CKs, to induce rooting or root formation. Sometimes this process can also be done ex vitro, by placing the different plantlets in pots with soil. Once grown plants are developed, they are acclimated by growing them in controlled environmental conditions prior to their transplantation into the field (Shahzad *et al.*, 2017; Haque *et al.*, 2022). An essential aspect of *in vitro* propagation is the preservation of aseptic conditions throughout the culture process to avoid contamination, which can not only impact the overall health of explants but also hinder shoot proliferation (Cassells, 1991; Park, 2021a).

In vitro propagation is a contributing factor on the development of new elite plant varieties through molecular breeding and genome editing. The combination of these techniques together with the multiplication speed and adaptability of *in vitro* propagation has allowed the generation of plants with relevant traits such as enhanced disease resistance, stress tolerance and improved yield (Gantait *et al.*, 2022). Furthermore, *in vitro* propagation can help preserve valuable genetic resources free of contaminations that can later be used to introduce relevant traits into new cultivars. Altogether, *in vitro* propagation has the potential of helping overcome the agricultural and environmental challenges resulting from an ever-growing population and climate change.

1.3.2 Callus culture

Callus is an amorphous mass of unorganized totipotent cells. This mass of cells can form when a plant is wounded or in the presence of pathogen infection, as a protective response aiming to seal off the damaged tissue or wounded area. This formation of callus in response to wounds or damage has been observed in almost all groups of plants (Evans, Coleman and Kearns, 2003a). In terms of tissue culture, callus is normally formed by culturing a part of a plant (explant), like for example a leave or a root, in a callus-inducing medium. This medium presents a high concentration of auxins, most commonly 2,4-D (2,4-dichlorophenoxyacetic acid), together with a very low or absent level of CKs. This combination of PGRs induces the dedifferentiation and proliferation of cells, which will ultimately lead to the formation of callus. Wounding the explant used for callus induction, for example by producing small incisions or sections, can sometimes enhance or fasten the formation of callus, mimicking the natural response of plants to damage. During dedifferentiation, cells undergo considerable structural and metabolic changes, including the activation of genes involved in cell division and the loss of chloroplasts (Bidabadi and Jain, 2020; Long *et al.*, 2022). Once callus is formed, the ratio of phytohormones present in the culture media can be used to exploit the totipotency of its cells, which allows the regeneration of specific plant tissues or even a full plantlet. Additionally, callus can be used to generate embryos in a process known as somatic embryogenesis, since embryos are formed from somatic cells (Evans, Coleman and Kearns, 2003a; Ikeuchi, Sugimoto and Iwase, 2013). While there is no official classification of types of calli, they can be divided according to some of their characteristics. In terms of aspect or physical consistency, calli can be ordered in compact (hard, dense and tightly packed mass of cells) or friable (soft and easily dispersible mass of cells) (Evans, Coleman

and Kearns, 2003a). Further, calli can also be divided according to their ability to form specific organs or tissues. Embryogenic callus can differentiate into embryos, while non-embryogenic cannot (Ikeuchi, Sugimoto and Iwase, 2013). Additionally, calli can be sorted into primary or secondary calli according to their origin. Primary callus is the one used to start a tissue culture and directly derives from an explant, while secondary callus is the result of a subculture or passing into fresh medium (Evans, Coleman and Kearns, 2003a).

Regarding subculturing, callus can be maintained on solid media for long periods by regularly passing it into fresh media (Evans, Coleman and Kearns, 2003a). Extended subculturing or passing comes with a cost, affecting the genetic stability of callus and leading to somaclonal variation. This phenomenon translates into genetic and epigenetic modifications that can result in phenotypic variations in the regenerated plants. In other words, these plants would not be genetically identical to the original starting material used to establish the culture (Evans, Coleman and Kearns, 2003b). While this variation can be used in plant breeding to generate plants with novel traits, this variability is unwanted when uniformity is important, like in the case of commercial propagation (Efferth, 2019). To counteract this, reducing the number of subcultures or passages together with routine checks of the genetic background of regenerated plants can be used, such as the use of specific markers for particular traits, to ensure that only true-clones are further kept (A. Hussain *et al.*, 2012; Md. S. Hussain *et al.*, 2012).

Callus cultures serve as a prevalent starting material for CRISPR-Cas and other precise genome editing technologies due to its ease of transformation, capacity for clonal propagation, and totipotency. Furthermore, some callus types, like friable or embryogenic one, possess high transformation ratios due to their easy infection through *Agrobacterium tumefaciens* (also sometimes taxonomically referred to as *Rhizobium radiobacter*). Additionally, the high proliferation and controlled *in vitro* growth can aid in increasing the chance of obtaining edited explants. A single edited cell or mass of cells can be selected by growing calli in media containing herbicides or antibiotics like hygromycin, in combination with screening for visually observable traits like the presence of fluorescence due to the expression of proteins such as GFP. The callus identified as a positive transformant can be further propagated, thus multiplying the possibilities of regenerating a viable and edited explant (Evans, Coleman and Kearns, 2003c; Purwantoro *et al.*, 2022).

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While calli cultures present promising approaches for commercial production of compounds and for generating new crops varieties when used in combination with new

genomic techniques, the low regeneration rates of plants remain a key bottleneck of calli cultures. This exemplifies the need for better understanding the molecular mechanisms underlying it. Together with advancements in media composition and culture conditions, knowing the regulation of the key processes involved in cell differentiation can help overcome this challenge (Efferth, 2019).

1.4 Plant genome editing

Genome editing (GE) refers to the insertion, deletion or replacement of nucleic acids in the genome of an organism. While researchers have been able to modify genomes for decades, a new set of tools with higher specificity, known as New Genomic Techniques (NGTs), have been developed during this century (Broothaerts *et al.*, 2021). In plants, these NGTs provide the ability of creating precise modifications that can lead to the improvement of traits which can directly impact agriculture and food security (Abdallah *et al.*, 2022). Over the past decades, these technologies have evolved significantly, advancing from the induction of non-specific mutations to the ability of modifying specific nucleotides and even multiplex editing, i.e., modifying several genes or targets at once. These tools have the potential to not only speed up genomics studies, but also to fasten crops improvement (Ahmad *et al.*, 2021). The three most used GE technologies in plants are Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-based systems (Li *et al.*, 2024). Most of these technologies rely on generating a double-stranded break (DSB) in a specific region of the targeted genome, using enzymes known as sequence-specific nucleases (SSNs) or site-directed nuclease (SDNs), that leads to the generation of mutations when the cleavage is repaired. There are two naturally occurring DNA repair mechanisms—non-homologous end joining (NHEJ) and homology-directed repair (HDR)—which can be exploited for different GE purposes (Gaj, Gersbach and Barbas, 2013). NHEJ is the predominant DNA repair mechanism in plant and mammalian cells, due to its independence of a homologous template and speed of repair (Mao *et al.*, 2008; Razzaq *et al.*, 2019). However, these traits make NHEJ an error-prone mechanism that directly ligates the ends of the cleaved strands, which often leads to the insertion or deletion of nucleotides (indels) close to where the DSB occurred. Such indels can lead to the creation of frame-shift mutations, which in turn can induce the formation of premature STOP codons. These changes can result in a gene knockout or a short peptide, which can be employed when aiming to obtain loss of function mutations (Devkota, 2018). HDR, on the other hand, relies on the presence of a template with homologous DNA sequences that flank the location of the DSB. This mechanism can be used to introduce single nucleotide changes, or to insert one or several transgenes (Gaj, Gersbach and Barbas, 2013). Although HDR can allow more accurate modifications, it is not a very common mechanism in somatic cells. While NHEJ is active in all cell cycle phases, HDR only happens in the S/G2 stages. This makes HDR more effective for GE purposes in germ-cells, where meiosis is active and can be exploited to induce homologous recombination of a donor or template DNA sequence (Devkota, 2018).

Tools like CRISPR-Cas9 have already proved their utility in generating crop variants with enhanced yield and improved tolerance to abiotic and biotic stresses. In fact, more than 40 crops, including important members of the grasses' family such as rice, maize, and wheat have already been the subject of trait improvement through genome editing (Abdallah *et al.*, 2022). For example, the introduction of a native GOS2 promoter in the untranslated region of the *ARGOS8* gene in maize, generated plants with improved

flowering and grain yield under drought conditions and without yield reduction under normal watering (Shi *et al.*, 2017). Similarly, the multiplex edition of quantitative trait loci (QTLs) associated with grain traits, have been targeted to improve grain yield in rice. Knocking out three genes, *OsGS3*, *OsGW2* and *OsGn1a*, previously identified as negative regulators of grain weight, size and number, led to an improvement of those same grain traits (Zhou *et al.*, 2019). Moreover, the recent development of tools capable of modifying specific nucleotides, i.e., Base Editing (BE), has led to the generation of varieties with enhanced traits without the need for the integration of exogenous genetic material or transgenes (Bharat *et al.*, 2020). It is known that many relevant agronomic traits are the result of single nucleotide polymorphisms (SNPs) in QTLs, which have been identified in big part thanks to genome-wide association studies (Zong *et al.*, 2017). A BE approach aimed to modify a base of the gene *NRT1.1B* in rice, translated in improved nitrogen efficiency on the modified plants (Lu and Zhu, 2017; Zong *et al.*, 2017). This gene encodes a nitrogen transporter, and a SNP where the replacement of a C base with a T base can lead to higher nitrate absorption (Hu *et al.*, 2015).

Plant genome editing can be divided, generally, in different steps. After a gene of interest (GOI) is identified, an appropriate GE tool must be selected according to the desired outcome of the edition (such as gene knockout or modified gene regulation), and that is compatible with the delivery method and transformation material that will be used (Gao, 2021). In many species, callus is widely used as the material for genome editing purposes, either being directly transformed or to regenerate a plant. However, the long time required to induce, establish, and propagate callus, along with the need to successfully transform and then regenerate an edited plant, are key restrictions of GE applications in plants. To counteract these limitations, researchers have begun utilizing several morphogenic factors, which are genes that target and regulate meristem and embryo development, to improve editing efficiency and enhance calli's ability to regenerate a transformed plant (Chen *et al.*, 2022). The use of factors like WOX and WUS holds substantial potential for engineering crops, particularly given the recalcitrance to transformation and regeneration encountered in certain monocot species such as perennial ryegrass (Chen *et al.*, 2022).

After transformation, there is a need for identification of edited explants or callus. This is commonly done by subjecting the transformed material to selective pressure using antibiotics. If an explant or callus has been successfully transformed, it will harbour resistance to the selecting agent through the integration of a transgene (Gao, 2021). Furthermore, plant genome editing can also be applied using stable or transient transformation methods, depending on whether the editing reagent used is integrated or not into the genome of the transformed cell or organism (Ran, Liang and Gao, 2017). Broadly speaking, transient transformation systems are those in which the introduced editing reagent is temporarily expressed, normally quickly degraded and does not integrate into the genome of the transformed cells, due to which the foreign material introduced in the cells is not inheritable by future generations. Conversely, in stable transformations the editing reagents are integrated into the genome of the targeted cells and are heritable by the progeny, which means that they can be eliminated by segregation processes (Gu, Liu and Zhang, 2021; Kocsisova and Coneva, 2023). Additionally, the integration of the editing reagents can lead to undesired mutations and mosaicism or chimerism (Nadakuduti and Enciso-Rodríguez, 2021). Transient methods are commonly used for functional studies, such as protein-protein interaction assays, or to perform screening experiments to determine the efficiency of editing reagents (Tyurin

et al., 2020). For cereal crop improvement where heritable edits are required, delivering the editor to germline or regenerable tissues is key. While stable transformation is often preferred due to efficiency and existing pipelines, heritable and transgene-free edited plants can also be obtained by segregating away the transgene or using virus-induced gene editing; thus, stable transformation is common but not strictly required (Wang *et al.*, 2019; Li *et al.*, 2020; Awan *et al.*, 2024; Qiao *et al.*, 2025).

In summary, genome editing has been and can be used for diverse applications, ranging from gene function studies to crop improvement. This section of the literature review covers one of the major genome editing techniques used in plants, CRISPR-Cas, providing an overview of its molecular mechanisms and delivery systems.

1.4.1 CRISPR-Cas systems

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems originated from the adaptive immune system of some bacteria and archaea, which they utilize to recognize and cleave exogenous DNA, particularly from phages (Malzahn et al. 2017). Bacteria can use CRISPR associated proteins (Cas) to cleave small fragments of exogenous DNA molecules, which can then be integrated into the CRISPR locus of the bacteria. This locus is normally formed by genes encoding Cas proteins and other accessory genes, together with a collection of CRISPR fragments that are separated from each other by variable DNA sequences known as spacers (Koonin and Makarova 2019). These short CRISPR repeats can act as a sort of library that enables bacteria to recognize and target future infections. For this, small fragments of RNA, known as CRISPR-derived RNAs or crRNAs, are transcribed from the previously integrated exogenous DNA sequences. The crRNAS are used to form surveillance or effector complexes, together with endonucleases such as Cas9, to monitor the presence and mediate the destruction of foreign DNA complementary to the crRNAs (Wiedenheft et al. 2012). The CRISPR repeats were first observed in *Escherichia coli* (Ishino et al. 1987), but their function remained unknown for decades. Similar repeats were identified in other bacteria and archaea, where it was observed that these fragments were identical to the sequences of viral DNA. This led to hypothesize that the CRISPR fragments could be used by bacteria or archaea to target specific viral DNA sequences, thus providing a "memory of infection", similar to the eukaryotic adaptative immunity based on RNA interference (Bolotin et al. 2005, Mojica et al. 2005, Pourcel et al. 2005, Makarova et al. 2006).

CRISPR-Cas systems are classified into two primary classes, differentiated by the architecture of their effector complexes. Class 1 systems are characterized by multisubunit protein complexes that facilitate their functions, whereas Class 2 systems operate through a single, multidomain effector protein. Each class is further divided into specific types and subtypes, based on features such as particular Cas proteins, locus organization, and target specificities. For example, the widely known and used CRISPR-Cas9 system belongs to the Type II system within the Class 2 (Koonin and Makarova, 2019). In the case of CRISPR-Cas9, the effector complex is formed by a double RNA structure made of the crRNA and a trans activating CRISPR RNA (tracrRNA) (Liu *et al.*, 2020). The tracrRNA is complementary to the sequence of a particular crRNA, and it is involved in the processing of the pre-crRNA into mature crRNA by triggering the activity of ribonuclease III (Koonin and Makarova, 2019). This dual RNA structure recognizes specific DNA sequences through two key elements: complementarity between the bases of the crRNA and the target DNA, and the presence of a protospacer adjacent motif (PAM) located downstream of the complementary region on the target DNA. This PAM sequence varies

depending on the Cas protein present in the effector complex, and it is 5'-NGG-3' in the case of Cas9 (Liu *et al.*, 2020). This endonuclease is formed by two cleavage or nuclease domains, the HNH and the RuvC-like domains, that allow it to cleave both strands of DNA 3 to 4 bp upstream from the PAM region, generating blunt ends. In a now famously known *Science* publication, Jinek and colleagues (Jinek *et al.*, 2012) showed that the effector complex from this bacterial immune system could be engineered to recognize and cleave specific DNA sequences. By coupling the Cas9 protein of *Streptococcus pyogenes* (SpCas9) with a synthetic RNA molecule, known as guide RNA (gRNA), the researchers were able to induce DSBs into specific regions of the *E. coli* genome. This gRNA was a chimeric RNA structure formed by the tracrRNA sequence of SpCas9 and a synthetic RNA molecule complementary to a desired DNA region (commonly referred to as gRNA or sgRNA). The gRNA comprised 20 nucleotides adjacent to a PAM sequence, excluding said motif, present in the target region (Jinek *et al.*, 2012). This discovery, which resulted in two of these researchers being awarded the 2020 Nobel Prize in Chemistry (*The Nobel Prize in Chemistry 2020 - Popular information*, no date), demonstrated that CRISPR-Cas could be utilized for precise genome editing (GE) purposes.

A key factor in the extensive use of CRISPR-Cas for plant genome editing experiments is the ease of synthesizing gRNA sequences, as DNA oligonucleotides, to target specific DNA regions. This simplicity, together with a lower cost of assembly and use, have made CRISPR-Cas systems the most widely used tool for genome editing in plants (Yin, Gao and Qiu, 2017; Gao, 2021). CRISPR-Cas systems are commonly used to induce knockout mutations in genes of interest, to study their function, or to generate organisms with specific traits or phenotypes. Additionally, CRISPR-Cas based tools can also be used for more complex applications such as gene knock-ins (introducing a gene coding sequence) and to regulate gene expression by for example targeting the promoter region of a gene (Yi Zhang *et al.*, 2020).

One of the main drawbacks of CRISPR-Cas methods is the occurrence of cleavage in unintended regions of the genome (Hwarari *et al.*, 2024). These edits, known as off-targets, are caused by homology between the gRNA and genomic regions different from the intended target (Manghwar *et al.*, 2020). Off-targets can have severe adverse effects, ranging from loss of function of edited genes to the emergence of detrimental traits or phenotypes (Zhang *et al.*, 2021). Similar to how gRNAs can be designed using several sets of informatic tools, such as *CRISPOR* (Concordet and Haeussler, 2018) or *Cas-Designer* (Park, Bae and Kim, 2015), off-targets can also be predicted in silico through tools like *Cas-OFFinder* (Bae, Park and Kim, 2014). In both cases, the bioinformatics tools rely on well sequenced reference genomes to provide valid outputs. When such reference is available, these predictors can provide specific genomic locations that can be later analysed to detect if the predicted off-targets have indeed been induced by the CRISPR editing systems, thus eliminating the need to use whole genome sequencing (Hajiahmadi *et al.*, 2019; Manghwar *et al.*, 2020). Additionally, knowing the sequences where the off-targets can happen, allows the modification of the gRNAs bases to minimize their occurrence. For example, selecting gRNAs with mismatches in the seed region (the 10 to 12 nucleotides proximal to the PAM region) can greatly reduce the chance of generating indels in this unintended location (Young *et al.*, 2019). Similarly, the GC content in the gRNA needs to be considered and kept in a moderate range (between 40 and 60 %) to not interfere with the proper CRISPR-complex activity (Bortesi *et al.*, 2016; Hajiahmadi *et al.*, 2019; Young *et al.*, 2019). Furthermore, Cas proteins, modified to have an enhanced specificity, have successfully been used to reduce the off-target activity of the nucleases.

These high-fidelity enzymes, such as eSpCas9 and SpCas9-HF, present point mutations that reduce the stability of the CRISPR-Cas complex with the target DNA when mismatches appear (Zhang *et al.*, 2018).

Different variations of CRISPR-Cas systems have been adapted for genome editing purposes. These variations are based on using different Cas proteins, such as Cas13 which induces DSBs in RNA molecules instead of DNA, or on modifying the activity of these enzymes, like using the dCas9 nuclease whose cleavage activity has been impaired and therefore allows binding of the CRISPR-complex to the DNA without inducing DSBs (Chen *et al.*, 2019). Using deactivated Cas nucleases has allowed to engineer the transcription and post-transcription regulation of genes (Lowder *et al.*, 2015; Tuncel *et al.*, 2025).

Moreover, by inactivating one of the nuclease or cleavage domains of the Cas9 protein, it is possible to cleave or nick only one strand of DNA, resulting in modified nucleases known as nickases or nCas9s. These nickases allowed the development of CRISPR-Cas based systems capable of modifying specific bases or nucleotides, such as Base editors and Prime editors (Lee *et al.*, 2023). Base editors (BEs) were originally constructed by fusing a dCas9 together with a deaminase enzyme, capable of small nucleotide conversions (e.g., converting cytosine to thymine or adenine to guanine) without the need of generating a DSB (Komor *et al.*, 2016). Replacing the dCas9 with an nCas9 that nicks the non-edited DNA strand, can enhance the editing efficiency of base editors by stimulating the endogenous DNA repair mechanism of cells to use the edited strand as a template for DNA repair (Li *et al.*, 2024). Prime editors (PEs) are a further refinement of this precise editing technology, where a viral reverse transcriptase (M-MLV RT) is combined with an nCas9 and a modified gRNA known as "prime editing gRNA" (pegRNA) (Anzalone *et al.*, 2019). The pegRNA contains the desired DNA modifications or edits downstream from the DNA recognition region, thanks to which it can act as a template for the reverse transcriptase activity. When the nCas9 nicks the target DNA, the reverse transcriptase uses the template present in the pegRNA to introduce specific nucleotide modifications in either the target or non-target DNA strand, including base conversions and indels without the need of a DSB (Anzalone *et al.*, 2019; Li *et al.*, 2024).

Figure 1 provides an overview of the mechanisms of the various CRISPR-Cas systems previously discussed. The CRISPR-Cas9 complex can be used to target a specific region of the genomic DNA through a complementary gRNA to induce double-strand breaks. This type of system can be used for either precise repair experiments exploiting the homology-directed repair (HDR) mechanism by providing a template or donor DNA or to induce random nucleotides insertions or deletions (indels) through the non-homologous end joining (NHEJ) repair mechanism (Figure 1A). Base editing (Figure 1B) and Prime editing (Figure 1C) rely on the use of a modified Cas9 nuclease capable of only breaking one of the DNA strands, known as nickases. Base editors can be used to induce specific nucleotide changes by fusing different enzymes such as deaminases and glycosylases to a nickase and rely on the specificity of the gRNA to DNA complementarity (Figure 1B). Prime editing is based on the fusion of a nickase with a viral reverse transcriptase. A prime editing gRNA (pegRNA) provides the specific interaction with the targeted genomic DNA together with a template for the reverse transcriptase. Once the nickase breaks the non-target DNA strand, the transcriptase can use the template to generate the desired mutations that will be incorporated into the genomic DNA after the DNA is repaired (Figure 1C).

Base editors and Prime editors are valuable tools when aiming to produce precise point mutations (Razzaq *et al.*, 2019) and have been effectively used to generate plants

with desired traits. For example, base editing was used to induce a point mutation in the rice alfa-tubulin gene, *OsTubA2*, to create mutant plants resistant to the herbicide dinitroaniline (Liu *et al.*, 2021). In the case of prime editing, researchers introduced an amino acid change in the *OsALS* (acetolactate synthase) gene of rice to confer tolerance to imidazolinone herbicides, which inhibit ALS activity (Zong *et al.*, 2022).

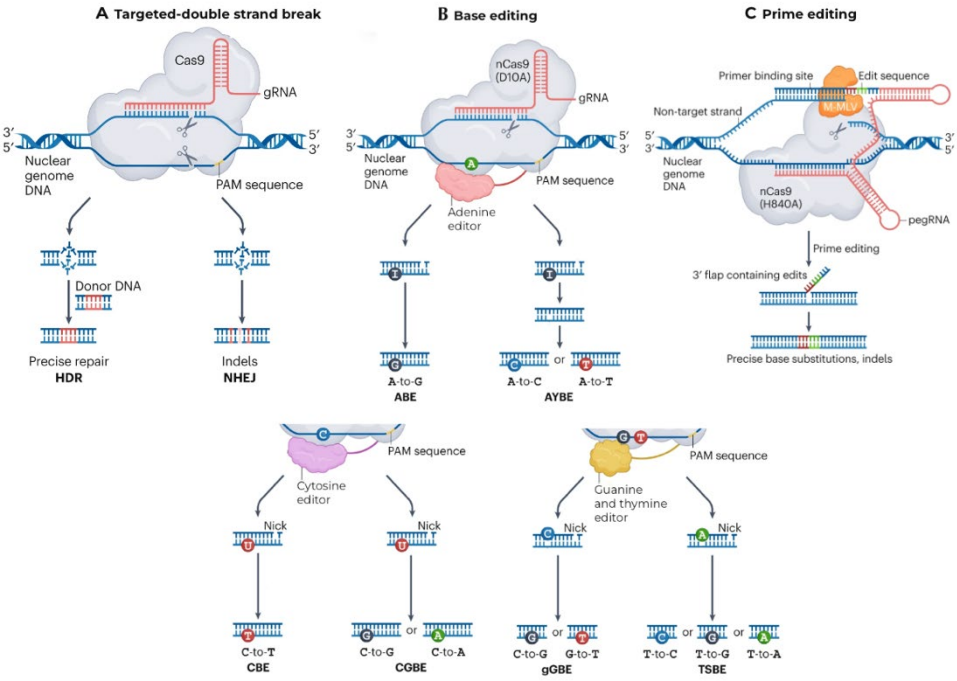

Figure 1 CRISPR-Cas editing systems. A *Double strand breaks (DSB) can be induced by using a CRISPR-Cas9 complex. The DSB can be exploited to generate random nucleotide mutations (indels) through the error-prone non-homologous end joining (NHEJ) DNA repair mechanism. Alternatively, if a donor or template DNA is provided, the DSB can be repaired through the precise homology-directed repair (HDR) mechanism.* ***B*** *Base editors are based on the fusion of nickases (nCas9) together with enzymes such as deaminases (adenine editor and cytosine editor, in red and pink respectively) and glycosylases (guanine and thymine editor in yellow). The nickase can only induce single strand breaks in the genomic DNA. The adenine editor (in red) can induce the change of A to G (ABE, adenine base editor) or A to C or T (AYBE, adenine transversion base editor) by using different deaminases. The cytosine and guanine editor shown here only present the enzyme responsible for the nucleotide conversion, since the fused Cas9 + gRNA complex is the same as depicted in the adenine editor. The cytosine editor (in pink) can induce the change C to T (CBE, cytosine base editor) and the changes C to T or A (CGBE, C-to-G base editor) by utilizing different enzymes. The guanine and thymine editor (in yellow) can induce the change C to G or G to T (gGBE, glycosylase-based guanine base editor) together with changes of T to C, T to G or T to A (TSBE, T-to-S (G/C) base editor).* ***C*** *Prime editing relies on the use of a nickase fused to a viral retrotranscriptase (in orange). The system is directed to a specific region of the genomic DNA by the prime editing gRNA (pegRNA) which also provides a template for the transcriptase to introduce the intended nucleotide changes that will be incorporated in the target site after the DNA repair mechanism mends the single strand break produced by the nickase. Figure modified from (Li* et al.*, 2024).*

1.4.2 Delivery systems and transformation methods

A key aspect to ensure the effectivity of plant genome editing is the efficient delivery of the genome editing reagents into plant cells. One of the main obstacles that these systems face is the presence of a cell wall that is absent in animal cells (Li *et al.*, 2024). Different delivery methods have been developed, the choice of which depends on the plant material that is going to be transformed, the reagents used for this purpose, and the aim of the expected edition. The three main delivery methods used to transform plants are: *Agrobacterium*-mediated transformation, through which DNA can be transferred into the plant cells; protoplasts transfection, which involves the delivery of editing reagents into cells that have been stripped of their cell walls; and particle bombardment (biolistics), which consists into shooting inert particles coated with the desired editing reagent into the target cells (Chen *et al.*, 2019, 2022). Generally, delivery systems are classified into direct or indirect methods, according to how the editing reagents are transferred into the cells. To overcome some of the limitations of these three main delivery techniques, new systems have been developed. For instance, delivering the editing reagents through plant viruses has the advantage of offering the possibility to avoid the use of tissue culture to obtain edited plants (Zhu, Li and Gao, 2020). One restriction of the original virus-assisted or induced gene editing (VIGE) is the inability of viruses to transfer large cargo (Li *et al.*, 2024). As most plant RNA viral vectors, which are positive-strand RNA viruses, cannot stably carry the SpCas9 cargo, early and prevailing VIGE approaches delivered only guide RNAs into plants already expressing the nuclease (Zhu, Li and Gao, 2020; Laforest and Nadakuduti, 2022; Shen *et al.*, 2024). To overcome this cargo limitation, viruses have been engineered to be able to deliver complete CRISPR-Cas complexes, including base editors (Li *et al.*, 2024). Moreover, co-delivery of Cas9 with guides is possible with certain negative-strand RNA viruses and with geminivirus DNA replicons (Shen *et al.*, 2024). Additionally, classical VIGE approaches were only able to induce transient gene expression, which as previously explained limits the hereditability of the edits. To overcome this, adding mobile RNA elements to the gRNA sequence, such as tRNAs, can facilitate the delivery of the CRISPR-Cas complex into the meristematic region, which helps to obtain heritable mutations (Ellison *et al.*, 2020; Zhu, Li and Gao, 2020).

In the case of CRISPR-Cas based systems, three main editing reagents are used: DNA, RNA and ribonucleoproteins (RNPs). These latter, RNPs, are preassembled complexes formed by purified Cas proteins and gRNAs. These complexes can be directly introduced into a cell without using foreign DNA, allowing DNA-free genome editing, which avoids the integration of transgenes in the edited organism (Ran, Liang and Gao, 2017; Gao, 2021). The following sections focus on two of the delivery methods utilized in conjunction with CRISPR-Cas based editing reagents: *Agrobacterium*-mediated transformation and protoplasts transfection.

1.4.2.1 *Agrobacterium*-mediated transformation

One of the first developed and most used delivery methods in plant genome editing is based on *Agrobacterium tumefaciens* mediated transformation (Mall *et al.*, 2018). During the 70s, it was discovered that this pathogenic soil bacterium was responsible for the development of crown gall disease through the introduction of part of its own genetic material, known as transfer DNA (T-DNA), into infected cells (Larebeke *et al.*, 1974; Zaenen *et al.*, 1974; Chilton *et al.*, 1977). This transfer of DNA is mediated by a tumour inducing plasmid (or Ti plasmid) that carries the T-DNA, which is the only portion of this plasmid that is transferred into the plant cells (Chilton *et al.*, 1977). The T-DNA contains

two sets of oncogenes: those responsible for the formation of the crown galls and those responsible for the synthesis of opines (Bourras, Rouxel and Meyer, 2015). Crown galls are tumour-like growths or swellings where the bacteria can grow and reproduce. For this purpose, the bacteria also integrate genes encoding opine synthases, which allows *A. tumefaciens* to modify the metabolism of cells and to induce the synthesis of opines, carbohydrate derivatives, that are used as a food source by the bacteria (Gelvin, 2003). The T-DNA is flanked by border sequences, which are the target of the virulence genes (*vir* genes). These *vir* genes encode different proteins that control the processing and transfer of the T-DNA (Gelvin, 2003). For example, VirD1 and VirD2 are nucleases responsible for the nicking of the T-DNA from the Ti plasmid into a single stranded DNA molecule or T-strand that will be transported into the host cell. Another protein, VirE2, contains nuclear localization signals that control the import of the single stranded T-DNA into the nucleus of the cell where it will be integrated into the plant genome (Bourras, Rouxel and Meyer, 2015).

The ability of *A. tumefaciens* to transfer DNA into plant cells has been exploited and refined for genome editing purposes. For this, modifications have been made into *A. tumefaciens* own genome, in the Ti plasmids and into the T-DNA itself (Gelvin, 2003). The first attempts to engineer the natural DNA transfer ability of *A. tumefaciens* for plant genome editing consisted into cloning a specific gene of interest (GOI) into the Ti plasmid. These experiments, while successful in inserting a GOI into the plant genome (Ruvkun and Ausubel, 1981; Zambryski et al., 1983), showed that the direct modification of the Ti plasmid was too complex (Gelvin, 2003). A different approach aimed at having the T-DNA containing a GOI and the vir genes in different replicons. Previous research had shown that when the T-DNA and the vir genes were in different replicons inside the same *A. tumefaciens* cell, the vir genes were still able of processing and transferring the T-DNA into infected cells (de Framond, Barton and Chilton, 1983; Hoekema et al., 1983). This led to the development of the binary vector system, in which a plasmid containing the vir genes (known as the helper plasmid) controlled the transfer of the T-DNA encoding the GOI from a separate plasmid, designated as the binary vector (Hoekema et al., 1983). Helper plasmids have been modified to not contain the T-DNA region, to eliminate the tumour inducing ability, and to be stably expressed by *A. tumefaciens* strains. In this manner, only binary vectors need to be engineered. Binary vectors can encode antibiotic resistance outside the T-DNA region, which can assist in cloning the vector into *E. coli* and when transforming *A. tumefaciens* to express said vector. Additionally, including antibiotic resistance coding sequences into the T-DNA region can be used for the selection of positively transformed cells. Similarly, fluorescent proteins can also be included in this region to assist in the screening of transformants (Lee and Gelvin, 2008). Binary vectors have revolutionized the ability of introducing foreign genes into plant genomes and are the basis of the *Agrobacterium*-mediated transformation used nowadays (Gelvin, 2003).

Using *Agrobacterium* as a delivery system for editing agents, such as CRISPR, is relatively simple and cost-effective. That, together with the ability to transfer large DNA fragments into plant cells, is useful when aiming to perform multiplex editing. The relatively low-copy number integration of the T-DNA region also underlines why this system is one of the most widely used delivery or transformation methods (Chen *et al.*, 2022; Ebrahimi and Hashemi, 2024). However, *A. tumefaciens* can only effectively infect a narrow number of plants species. Some monocot plant species are particularly recalcitrant to *Agrobacterium*-mediated transformation or have very low regeneration efficiency when used in tissue culture procedures, such as callus induction and transformation (Ebrahimi

and Hashemi, 2024). Different initiatives have aimed towards overcoming this limitation. Some researchers have studied and tested the use of morphogenic genes and growth factors to improve the transformation efficiency of monocot plants. For example, overexpressing the wheat gene *TaWOX5*, a member of the family of morphogenic genes *WUSCHEL*, improved the regeneration and transformation efficiency of wheat calli edited through *Agrobacterium*-mediated transformation. Significantly, also calli from recalcitrant wheat varieties were transformed and regenerated plants were obtained (K. Wang *et al.*, 2022). Similarly, improved regeneration and transformation have been achieved using a chimeric protein that combines a growth factor, GROWTH-REGULATING FACTOR 4 (GRF4), and its cofactor, GRF-INTERACTING FACTOR 1 (GIF1). Utilizing a binary vector encoding GRF-GIF and CRISPR-Cas9 in the T-DNA region, scientists were able to successfully regenerate wheat plants from embryos co-cultivated with *A. tumefaciens* (Debernardi *et al.*, 2020). Furthermore, the high importance of the *vir* genes in the processing and delivery of T-DNA has made them attractive candidates for improving the transformation efficiency of *Agrobacterium*-based delivery of editing agents (Gelvin, 2003). Two *vir* genes responsible for the initiation of the T-DNA transfer, *virA* and *virG*, can be inhibited by salicylic acid (SA). Some plants produce high levels of this acid in basal conditions, while others accumulate it in response to stressful conditions, such as the originated from *A. tumefaciens* infection. In both cases, the high levels of SA block *A. tumefaciens*-mediated transformation (Yuan *et al.*, 2007). To overcome this limitation, the phenolic compound acetosyringone can be used to counteract the inhibitory effect of SA (Chen *et al.*, 2022). Another method utilized to improve transformation efficiency consists in the development of super-virulent *Agrobacterium* strains harbouring modified *virG* genes (De Saeger *et al.*, 2021).

There are different methods used to exploit the DNA transfer ability of *Agrobacterium*. In most cases, a suspension that contains a specific bacterial strain, encoding the desired binary vector, is used to put the bacteria in contact with the plant tissues to be transformed. For example, in co-cultivation methods, calli or other plant material are immersed or covered with the *Agrobacterium*-containing suspension. This technique requires tissue culture and regeneration steps, which can be cumbersome (Niazian *et al.*, 2017). To avoid this, other methods rely on the direct delivery of *Agrobacterium* into plant cells. These include agroinfiltration, where the bacterial suspension is injected into the plant material or introduced by applying pressure (Rossi *et al.*, 1993; Schöb, Kunz and Meins Jr., 1997). A similar direct delivery technique is floral dip, which consists of immersing the inflorescences of a plant in a bacterial suspension that contains surfactants (Clough and Bent, 1998). Infiltration and floral dip are the most effective and commonly used methods in dicot plants, while co-cultivation is the preferential choice in monocot organisms such as *L. perenne* (Niazian *et al.*, 2017). Dicot plants are innately susceptible to *Agrobacterium* infection and typically possess simpler inflorescences, larger intracellular spaces, and thinner cell walls compared to monocot plants. (Bélanger *et al.*, 2024).

A. tumefaciens assisted delivery is mainly utilized for stable transformation events that use DNA based editing, since the T-DNA of the binary vector, and sometimes elements of the backbone too, is integrated into the genome of the host cells. This integration can limit the use of this delivery system when aiming to develop transgene free plants (Chen *et al.*, 2019). Nonetheless, *Agrobacterium*-mediated transformation remains an effective tool that has successfully been used to generate plants with improved traits through the delivery of CRISPR-Cas systems.

1.4.2.2 Protoplasts

Protoplasts, also referred to as “naked cells”, are cells whose cell wall has been removed, through either chemical or mechanical methods (Cocking, 1972). While protoplasts tend to be commonly associated with plants, cell-wall free cells can also be isolated from fungi and bacteria. Protoplasts can be obtained from diverse plant materials, including leaves, callus and embryos. The source from which protoplasts are isolated can determine the viability of the cells and their ability to be used for downstream applications such as plant regeneration (Mukundan, Satyamoorthy and Babu, 2025). A key aspect is the age of the source material, where younger plant tissues and materials tend to yield a higher number of viable protoplasts (Reed and Bargmann, 2021; Chen *et al.*, 2023; Mukundan, Satyamoorthy and Babu, 2025). In the case of monocot species, the choice of the optimal material depending on the goal or end use of the protoplasts is paramount. While protoplasts derived from callus or embryogenic cultures can be transformed and regenerated afterwards, the same does not apply for protoplasts whose origin is other tissues such as leaves or tillers (Lin *et al.*, 2018). Monocot plants have only small portions of meristematic tissue, where totipotent cells are located, and therefore capable of regenerating a full plant (Hu *et al.*, 2017; Lin *et al.*, 2018; Pasternak *et al.*, 2020; Pujari *et al.*, 2021). Due to this limitation, regeneration from monocot protoplasts is considered challenging and explains why in most cases protoplasts are used for transient transformation experiments (Lin *et al.*, 2018). Protoplasts can be generated relatively easily from tillers and leaves, making them ideal candidates for reverse genetics experiments, to study protein-protein interactions and as a screening platform to test the efficiency of editing reagents such as CRISPR-Cas systems (Chen *et al.*, 2023). For genome editing purposes, using protoplasts englobes three main steps or phases: isolation, purification and transformation.

The first isolation of protoplasts consisted in the use of a hypertonic solution to induce the plasmolysis of mesophyll cells, which led to the destruction of the cell wall, and the shrinkage and circularization of the resulting naked or cell-wall-less cells. Once the cells were plasmolyzed, the tissue from where they originated was cut to liberate the newly formed protoplasts. This first method was described in 1892 by the Swedish botanist John af Klercker, who used this technique to isolate protoplasts from the aquatic plant S*tratiotes aloides* (af Klercker, 1892). Chemical isolation methods are currently the most widely used, as mechanical methods typically yield fewer intact and therefore “useful” protoplasts (Davey *et al.*, 2010). This chemical approach is based on the use of enzymes capable of degrading the different components of cell walls, such as cellulase and pectyolase (Cocking, 1972). Edward C. Cocking described the use of a fungal cellulase to isolate protoplasts from the root tips of tomato seedlings, demonstrating the feasibility of chemical isolation methods for the obtainment of plant protoplasts (Cocking, 1960). Since then, several advancements have been done to this original method to enhance the yield and quality of the isolated cells. A key aspect of the chemical isolation of protoplasts involves using an appropriate enzyme solution. The types and concentrations of enzymes in this solution, as well as the duration of the enzymatic treatment, must be adapted to the specific plant material from which the cells are isolated. This is crucial because cell wall structure and composition vary not only among plant species but also between different plant materials and tissues, thus making the correct selection of enzymes essential (Mukundan, Satyamoorthy and Babu, 2025). Furthermore, using enzymatic solutions with a proper pH and osmotic stabilizers, can enhance the activity of the enzymes and preserve the integrity of the isolated cells (Chawla, 2009b). Protoplasts

isolation can be improved by pre-treating the source tissue with hypertonic solutions like mannitol to induce plasmolysis, facilitating wall digestion (Davey *et al.*, 2010). Enhancing enzyme activity can be achieved by partially damaging the tissue or applying vacuum to improve enzyme solution infiltration (Mukundan, Satyamoorthy and Babu, 2025). Moreover, gentle agitation can help separate protoplasts during enzymatic treatment (Reed and Bargmann, 2021). For optimal viability and yield, enzymatic treatment is often performed in the dark to prevent oxidative damage that can lead to cell bursting and hinder division and regeneration (Reed and Bargmann, 2021; Chen *et al.*, 2023).

Following isolation, viable protoplasts must be separated from cellular debris and chemical residuals (Chawla, 2009b; Davey *et al.*, 2010). The purification process involves washing the isolated protoplasts in a solution containing an osmotic agent, similar to that used during isolation, to prevent bursting (Chawla, 2009b; Davey *et al.*, 2010). This washing step is also crucial for diluting residual enzymes (Mukundan, Satyamoorthy and Babu, 2025). Washing is accompanied by filtration steps, normally using nylon meshes or metallic sieves, to remove big cellular debris. After filtration, the resulting suspension is centrifugated to eliminate the remaining cellular debris by discarding the supernatant (Chawla, 2009b; Davey *et al.*, 2010). Additionally, preforming a density gradient separation, dead and alive cells can be separated. The previously pelleted protoplasts are layered on top of a solution with a different density, which allows creating different phases separating cellular debris, dead cells and viable or alive protoplasts after centrifugation. For example, this can be achieved by layering the pelleted protoplasts on top of a sucrose solution, or cushion (Chawla, 2009b; Davey *et al.*, 2010; Chen *et al.*, 2023).

Protoplasts can be utilized for genome editing by directly delivering DNA-based editing reagents, such as binary vectors commonly used in *Agrobacterium*-mediated transformation (Yue *et al.*, 2021). Two of the most common transformation techniques used in conjunction with protoplasts involve the partial disruption of the cellular membrane of the cells to facilitate the penetration of the editing reagents (Chawla, 2009a). One such method is electroporation, in which a small and short electrical pulse is used to disrupt the cellular membranes, creating pores through which the editing reagents can enter the cells (Joersbo and Brunstedt, 1991). While this method is relatively simple to use, it requires specific equipment and can easily lead to bursting of the protoplasts if not well employed. An alternative technique relies on the use of polyethylene glycol (PEG), a chemical compound that can increase the permeability of cell membranes. When used in conjunction with calcium or magnesium ions, that promote membrane fusion, PEG-mediated transformation can be used to deliver editing agents into protoplasts (Dix *et al.*, 1988; Gharti-Chhetri *et al.*, 1992). Using PEG is relatively simple, does not require specific equipment and is better suited to deliver bigger reagents such as ribonucleoproteins (RNPs) (Bart *et al.*, 2006; Subburaj *et al.*, 2022). Unfortunately, PEG can damage the cells through chemical toxicity, hence affecting viability and downstream applications such as regeneration (Gharti-Chhetri *et al.*, 1992; Bart *et al.*, 2006). Successful transformation relies on the interaction between cells and editing agents (Ohyama, Gamborg and Miller, 1972; Larkin *et al.*, 1990). If the cell density is too low, the probability of editing agents interacting with the protoplasts is reduced. Conversely, if the cell number is too high, cells can cluster into aggregates and suffer from local osmotic stress, collectively reducing the viability of protoplasts and their interaction with the editing agents (Ohyama, Gamborg and Miller, 1972; Larkin *et al.*, 1990; Bergman and Glimelius, 1993; Jarl and Rietveld, 1996).

Therefore, using an appropriate cell density or number of cells is directly connected to the successful transformation of protoplasts (Reed and Bargmann, 2021).

Figure 2 depicts how protoplasts can be used in conjunction with CRISPR reagents. Different plant tissues can be used to isolate protoplasts, which can then be transformed with ribonucleoproteins or plasmids encoding CRISPR complexes. Transformation can be achieved by different methods, including electroporation and polyethylene glycol (PEG) based delivery. After successful transformation, DNA from the protoplasts can be extracted and analysed using bioinformatics tools such as TIDE to assess the editing efficiency (Brinkman *et al.*, 2014). The edited protoplasts can then be used to induce the formation of calli that can be used to regenerate an explant. The explants can be further genotyped, and those containing the desired edits can be passed into pots to harvest their seeds.

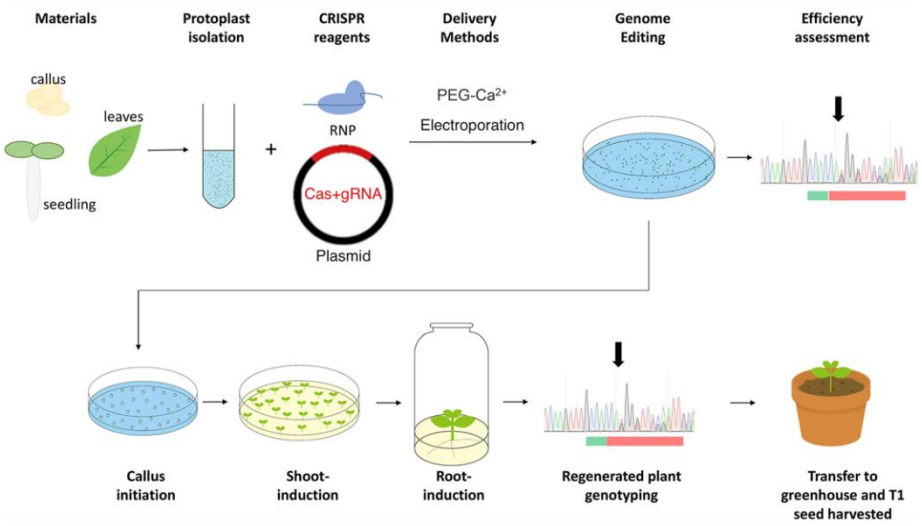

Figure 2 Use of protoplasts for CRISPR editing purposes. *The top panel shows different plant materials commonly used as the source for protoplasts isolation. Once protoplasts are obtained, they can be transformed with ribonucleoproteins or plasmids encoding the CRISPR-Cas complex (in red). The transformation can be done using different methods such as electroporation or by using polyethylene glycol (PEG) in conjunction with calcium ions. The transformed protoplasts can be used to determine the editing efficiency of the editing reagents, by analysing their extracted DNA with tools such as TIDE. In the bottom, edited cells can be cultured in specific media to induce the formation of calli, that can be later used to regenerate explants by successive subcultures in specific media. DNA from the regenerated plantlets can be used to genotype them, and those explants containing the desired edits can be passed into pots. Later, the seeds from the edited plants can be harvested to obtain a first generation of edited organisms (T1). Figure modified from (Yue* et al.*, 2021).*

Once transformation has been achieved and confirmed, protoplasts can be used to regenerate plants. Following transformation, protoplasts cultures are typically initiated in liquid media, then transferred to semi-solid media, and finally to solid media for the successful regeneration of plantlets or explants (Reed and Bargmann, 2021). These media contain a specific combination of nutrients and hormones (Chawla, 2009b). Liquid medium is usually used first because it provides a proper environment for cells to preserve their integrity and to start dividing and sometimes forming microcalli. These liquid media favour

the diffusion of nutrients into the cells, and contain sugars, such as mannitol and sucrose, that help maintain the osmotic balance and provide a carbohydrate source (Davey *et al.*, 2010). Since protoplasts lack a cell wall, the regeneration of said structure is vital for their survival and regeneration. After growing the cells in liquid media, semi-solid media can help the synthesis of cell walls by providing physical support and stability (Davey *et al.*, 2010). The semi-solid media normally contain a gelling agent, such as low-melting agar or alginate, which is used to form a single or multiple semi-solid layers creating a matrix that provides physical support for the cultured cells. Alginate is a gelling agent that solidifies when exposed to Calcium ions (Ca^{2+}). This characteristic makes alginate a common gelling agent when working with cells that are sensitive to high temperatures (Davey *et al.*, 2010). There are two main strategies when using semi-solid media for protoplasts regeneration: either embedding the cells in semi-solid media or placing the liquid media on top of the semi-solid matrix (Davey *et al.*, 2010). Additionally, the gelling agents can be used to form beads or droplets in which the protoplasts are embedded. For example, the liquid culture containing protoplasts can be mixed with alginate, followed by "dropping" this mix, using a pipette to create droplets, in a media containing calcium ions to form semi-solid beads (Davey *et al.*, 2010; Reed and Bargmann, 2021). To promote the growth, division, and subsequent regeneration of cultured protoplasts, nurse cultures can be used. These cultures involve co-culturing transformed protoplasts with actively dividing protoplasts, such as those derived from embryogenic calli, which can be from the same or a different origin than the transformed cells (Davey *et al.*, 2010; Reed and Bargmann, 2021). Nurse cells release growth promoting factors into the media, that can stimulate the division and growth of the transformed protoplasts, hence improving the following regeneration. To avoid mixing the two types of cells, it is recommended to physically separate both kinds of protoplasts (Davey *et al.*, 2010). Bead cultures of transformed protoplast can also be used in combination with nurse cultures, by placing the beads or droplets in liquid media containing the nurse cells (Reed and Bargmann, 2021). Semi-solid media provide a good environment for the formation of microcalli, that can be further grown by passing the cultures to solid media (Biswas and Zapata, 1990). Once the calli is established, the composition of the media can be changed to induce the formation of roots and shoots, ultimately leading to the regeneration of a complete plant (Biswas and Zapata, 1990; Reed and Bargmann, 2021).

While many advances have been done towards the efficient regeneration of protoplasts, this step is often difficult, particularly in the case of monocot species such as grasses, which underlines the use of protoplasts primarily to either test editing reagents or to perform essays whose goal is not to regenerate plants (Reed and Bargmann, 2021; Yue *et al.*, 2021). Nonetheless, the ability to deliver editing reagents different from DNA, such as RNPs, makes protoplasts attractive candidates to create transgene free plants if protoplasts with an intact totipotency are transformed by such means (Lin *et al.*, 2018). While the effective use of RNPs for editing protoplasts of important grasses has been reported, such as in rice (Zhang *et al.*, 2022), no plants have been regenerated through this approach.

2 Aims of the study

This doctoral thesis aimed to characterize the transcriptomic responses of plant genotypes with differing tolerance to drought and low temperature, to identify candidate genes for enhancing abiotic stress resistance in perennial ryegrass via genome editing. In addition, this research established a platform for evaluating genome editing agents targeting these genes, thereby supporting future genome editing implementation in *Lolium perenne*.

- To investigate the mechanisms underlaying differential tolerance towards drought in perennial ryegrass genotypes.

- To assess the genetic influence in low and freezing tolerance in perennial ryegrass genotypes with dissimilar stress tolerance.

- To generate a platform based on protoplasts transformation for testing the efficiency of editing agents (plasmids and gRNAs) *in vivo*, prior to their use in further genome editing applications in perennial ryegrass.

- To offer a comprehensive review of the basic mechanisms of different abiotic stresses in grasses and how genome editing can be used to provide plants with enhanced stress tolerance.

3 Materials and methods

3.1 Plant material

Different perennial ryegrass genotypes were used when performing the experiments mentioned in this thesis. To study the effect of mild drought stress on growth, two different genotypes of Lithuanian origin were analysed: a drought tolerant genotype ("3177") and a sensitive one ("3575") (Publication I). To uncover the mechanisms behind different tolerance towards freezing stress, two sensitive ("17-5" and "4-1") and two tolerant ("10-5" and "13-2") genotypes characterised in a different project were analysed (Publication II). Additionally, the Lithuanian perennial ryegrass cultivar Veja was used to isolate and transform protoplasts aiming to evaluate the editing efficiency of different gRNAs *in vivo* (Publication IV).

3.2 Methods

The methods mentioned below were employed during the completion of this doctoral thesis, with further information provided in the associated publications:

- Biomass accumulation evaluation through LER measurements – Publication **I**
- Physiological measurements (RWC and FV/Fm) – Publication **I**
- Electrolyte leakage measurement – Publication **II**
- RNA extraction and DNA digestion – Publications **I** and **II**
- Quality control, alignment, functional annotation and differential expression analysis of RNA sequences – Publications **I** and **II**
- Co-expression analysis of transcripts – Publication **I**
- Binary vectors construction using Golden Gate assembly – Publication **IV**
- Guide RNA design – Publication **IV**
- Protoplasts isolation and transformation – Publication **IV**
- Evaluation of protoplasts transformation efficiency through fluorescence detection – Publication **IV**
- CRISPR-Cas editing efficiency evaluation (TIDE sequencing analysis) – Publication **IV**

4 Results and discussion

To improve the text's flow and readability, the discussion sections from each publication have been integrated with their respective results.

4.1 Physiological effects of mild drought on perennial ryegrass (Publication I and Publication III)

To evaluate how mild drought affects genotypes with different tolerance towards the stress, three aspects were measured: leaf growth, relative water content (RWC) and photosynthetic activity (Fv/Fm).

Leaf growth can be used to assess biomass accumulation, which is one of the most important aspects that determine the yield of forage grasses such as *L. perenne* (Pittaro *et al.*, 2024). Leaf Elongation Ratio (LER) makes it possible to measure how leaf growth responds to water deficit (or increases in soil water potential) in a time-independent manner. Moreover, it allows connecting phenotypic responses to particular genotypes, by evaluating when drought induces leaf growth to decrease (Σ) or to completely stop (σ) (Yates *et al.*, 2019; Jaškūnė *et al.*, 2020). The studied drought tolerant genotype was able to maintain leaf elongation until the soil water potential (Ψ) reached 3.7 log10 hPa, when leaf growth decreased (Σ) and eventually ceased at 4.3 log10 hPa. Conversely, the leaf growth of the sensitive genotype started to slow down and stopped at lower Ψ levels of 1.6 log10 hPa and 1.8 log10 hPa, respectively (Publication I, Fig. 2A). These results showed that mild drought is harmful enough to detrimentally alter the biomass accumulation of the sensitive genotype, while the tolerant one was able to maintain leaf growth under the same conditions of water deficit and was only affected by higher levels of stress. Biomass accumulation is directly linked to the yield of forage grasses and can be determined by the ratio at which leaves grow or elongate. Evaluating the LER values of genotypes of interest can be used to assess their suitability to sustain drought periods, which can undermine and hinder the productivity of forage grasses since it directly impacts growth. When water is insufficient, plants employ different mechanisms to cope with the stress, aiming to preserve their cellular water content and prevent death. A common response of grasses to drought is to halt growth to mobilize resources and energy into other mechanisms that alleviate the lack of water (Zwicke *et al.*, 2015; Loka *et al.*, 2019; Lechowicz *et al.*, 2020) (Publication III). Determining this growth arrest is very valuable when evaluating grasses used for forage production. Plants that reach this point earlier than others will present reduced biomass accumulation, while those that reach it too late may suffer excessive drought damage and may not be able to resume growth once the stress subsides. Therefore, a balance must be struck when selecting grasses that can sustain growth under drought conditions, but that do cease it early enough when the stress worsens, hence being able to restart biomass accumulation once water deficit is absent. The moment at which plants perceive water deficit as a stressful condition can also be determined by observing when leaf growth begins to slow down (Σ). Given that the sensitive genotype exhibits this reduction in LER values earlier than the tolerant genotype, it can be inferred that the sensitive genotype senses and responds to drought stress sooner than the tolerant one. The water content and photosynthetic activity of a plant are directly connected to leaf elongation, therefore a decline on either of these factors has a direct impact in leaf growth (Fahad *et al.*, 2017).

A similar result to that of LER assessment was observed when evaluating the RWC of both genotypes under 5 days of mild drought (Publication I, Fig. 2C). To determine how

stressed a plant is by lack of watering, the RWC can be measured, since it correlates with the amount of water present in the tissues of the examined plant. As previously mentioned, one of the main impacts of drought is a reduction of the cellular water levels (Publication III). A sensitive plant would commonly show an earlier and more abrupt reduction in RWC than a tolerant one, since it lacks the ability to properly regulate its water content under water deficit (Huang, DaCosta and Jiang, 2014; Zwicke *et al.*, 2015; Loka *et al.*, 2019; Lechowicz *et al.*, 2020). Hence, RWC can point out when a plant is suffering from drought stress and can be used to compare organisms with differential phenotypic responses towards it. In the evaluated plants, while both genotypes presented a decrease in RWC with the progression of water deficit, the sensitive genotype had significantly lower levels at the end of the experiment (day 5), when comparing the same RWC measurements from the tolerant genotype. Furthermore, the RWC levels of the sensitive genotype decreased statistically significantly earlier, in the 4th day of the experiment, when comparing the RWC measured at the beginning of the experiment (day 1). This same comparison resulted in statistically significant lower RWC values only in the 5th day of the experiment in the case of the tolerant genotype. Thus, based on the RWC evaluation, it seems like under mild drought conditions the sensitive genotype presents symptoms of stress earlier than the tolerant. This correlates with the indication, derived from the LER measurements, that the sensitive genotype senses and responds earlier to mild drought.

Under drought stress, growth is also affected by stomatal closure, which aims to preserve the water content in plants tissues (Publication III). When stomata close, this directly impacts an organism's ability to capture CO_2 and generate carbohydrates through photosynthesis (Publication III). Therefore, reduced stomatal conductance lowers the photosynthetic activity, which in turn leads to reduced resources available to promote growth (Huang, DaCosta and Jiang, 2014; Zwicke *et al.*, 2015; Loka *et al.*, 2019). A comparable observation was reported when assessing the photosynthetic activity of the genotypes through the evaluation of maximum quantum efficiency of Photosystem II (Fv/Fm) (Publication I, Fig. 2D). Fv/Fm is a parameter commonly used to determine the maximum quantum efficiency of Photosystem II, which allows evaluating damage to the photosynthetic apparatus and photoinhibition under stressful conditions. When comparing basal or normal conditions against stressful ones, Fv/Fm tends to present reduced values under severe drought (Baker, 2008; Murchie and Lawson, 2013). Therefore, while mild drought is not expected to highly impact Fv/Fm values, reduced photosynthetic activity can be an indication of stressful conditions. Such is the case with the sensitive genotype evaluated in this publication, which presented reduced Fv/Fm values in the last days of the experiment (days 4 and 5) when compared to basal conditions. In addition, the tolerant genotype had no significant reductions in Fv/Fm values throughout the experiment and significantly higher values in the last day of the experiment when compared to the sensitive genotype. The stable Fv/Fm values recorded for the tolerant genotype may be an indication of photoprotective mechanism, such as nonphotochemical quenching, being active in this genotype (Baker, 2008). In conjunction, the Fv/Fm evaluation further supports the hypothesis that mild drought affected more the sensitive genotype. Additionally, this higher state of stress can hinder the growth of the sensitive genotype, since reduced photosynthetic activity correlates with lower leaf growth. Further evaluation of the photosynthetic activity of the sensitive genotype under prolonged drought conditions are needed to corroborate this hypothesis, since none of the Fv/Fm values

measured for this genotype were below the 0.75 threshold commonly used to define stress and damage in plants (Bolhàr-Nordenkampf *et al.*, 1989).

In summary, the evaluation of LER, RWC, and Fv/Fm indicates that the sensitive genotype senses and responds to drought earlier than the tolerant genotype. Furthermore, mild drought had a bigger impact on the sensitive genotype's physiology, affecting its ability to sustain leaf growth after 5 days.

4.2 Differential gene expression during mild drought underlying contrasting stress tolerance (Publication I and Publication III)

To evaluate the possible effects of mild drought on gene regulation, the samples from the first day of the experiment were used as controls (D1M) and compared against the expression levels of the samples collected under drought conditions (days 3 to 5), both in the morning and afternoon. The samples from day 2 were excluded from this and subsequent analysis, since it was too early in the experimental setup to observe differences among the genotypes caused by the stress. Additionally, samples were also collected at the end of the experiment, after rewatering the plants, to evaluate the genotypes' ability to recover from water deficit. These samples were contrasted against the ones corresponding to the last drought related sampling point, on the afternoon of day 5. The analysis of differentially expressed genes (DEGs) showed that a higher number of genes were differentially expressed in the sensitive genotype (13703) than in the tolerant one (3161) under mild drought and rewatering conditions. While 2632 of these DEGs were shared between both genotypes, the sensitive one had more upregulated and downregulated DEGs than the tolerant genotype in all comparisons, apart from the afternoon of day 3 and control conditions (D1M) contrasts (Publication I, Fig. 3C). Furthermore, when comparing the number of DEGs between morning and afternoon sampling points, the sensitive genotype had a higher number of DEGs in the morning, and the opposite was observed for the tolerant genotype (Publication I, Fig. 3C). Some of the genes deemed to be differentially expressed correspond to transcripts previously associated with drought response. For example, genes belonging to families of transcription factors (TFs) such as WRKY and MYB presented higher expression in the sensitive genotype under drought conditions than in the tolerant genotype. These specific TFs are commonly upregulated in response to drought stress, which suggest that the sensitive genotype was more affected by the studied mild drought conditions than the tolerant genotype. Conversely, genes related to cell growth were downregulated in the sensitive genotype, while their expression was less affected in the tolerant one. Once more, this points to the tolerant genotype being less affected by the studied mild drought than the sensitive.

Regarding the DEGs themselves, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses were performed to evaluate in which mechanisms and pathways they were involved. Comparing the different sampling points of both genotypes revealed that distinct pathways were enriched. Accordingly, the sensitive genotype showed a higher number of enriched pathways with up- and downregulated genes than the tolerant one during mild drought conditions, except for the afternoon of day 3. The sensitive genotype seems to respond to mild drought by upregulating genes related to sucrose metabolism and PGRs signalling (Publication I, Fig 4A); while simultaneously downregulating genes linked to DNA replication and motor proteins pathways (Publication I, Fig. 4B). Furthermore, comparing pathways enrichment

between morning and afternoon samples showed that plants of the same genotype had different transcription levels depending on the sampling time. In the case of the tolerant genotype, the samples belonging to the first day of stress presented a higher number of enriched pathways linked to upregulated genes than in the afternoon measurements. In this sense, the tolerant genotype showed enrichment of the photosynthetic pathway in the afternoon of days 3 and 5, while in the sensitive genotype this pathway was only enriched in the first mild drought sampling. This dissimilar pattern could relate to the sensitive genotype having reduced photosynthetic activity under mild drought, as shown by the reduced values of Fv/Fm (Publication I, Fig. 2D). Additionally, the enrichment observed only in the afternoon samples of the tolerant genotype could denote that this genotype had a delayed response to light stimuli. This could perhaps explain why the overall Fv/Fm values of the tolerant genotypes were slightly inferior to those observed for the sensitive genotype during the first 3 days of mild drought (Publication I, Fig. 2D). It is interesting to mention that the autophagy pathway was enriched in the set of upregulated genes in the sensitive genotype on the last day of mild drought, both in the morning and afternoon samples, but autophagy linked genes were downregulated in the sensitive genotype after rewatering (Publication I, Fig 4). This same pathway was not enriched at any timepoint in the samples from the tolerant genotype. Autophagy has been shown to be related to the degradation of molecules resulting from drought stress, both toxic and signalling components, together with the elimination of aquaporins aiming to reduce water loss (Tang and Bassham, 2022; Y. Li *et al.*, 2023). The upregulation of this pathway in the sensitive genotype further sustains the indication from LER measurements that this genotype has a stronger and earlier response to mild drought, leading to a more abrupt leaf growth arrest. This drastic response to the stress is probably related with a more severe impact of water deficit in the sensitive genotype than in the tolerant one. When assessing the pathways enriched in the last days of the experiment, the tolerant genotype had a higher number of enriched pathways on the last experimental sampling point than in control conditions. Together with the significantly lower RWC values than those observed in basal conditions, suggest that this genotype starts to be affected by water deficit after 5 days without watering.

The succession of days without watering also influenced the enriched pathways observed in KEGG analysis. While the tolerant genotype only showed enrichment of the Mitogen-activated protein kinase (MAPK) pathway after 4 days of mild drought, the sensitive genotype presented an enrichment one day earlier. Similarly, the sensitive genotype presented upregulation of genes related to the galactose metabolism sooner than the tolerant genotype, which upregulated genes linked to this pathway only on the last day of the experiment. MAPK signalling is highly involved in the responses of plants towards stress, where they interact and integrate the activity of other molecules such as ABA. Some of these drought related responses include stomatal closure, osmolyte accumulation and growth suppression (Majeed *et al.*, 2023). The high expression of genes linked to the MAPK pathway in the sensitive genotype may promote growth arrest, which would relate to the LER measurements recorded for these plants. This observation further consolidates the hypothesis of a more stressful state and stronger drought response in the sensitive genotype, leading to earlier leaf growth cessation. Galactose accumulates in response to drought, aiming to stabilize cellular functions and to preserve osmotic conditions. In perennial ryegrass, the upregulation of galactinol synthase (GOLS) has been shown in response to drought. GOLS can use galactose to synthesize galactinol, which in turn allows plants to make raffinose and other related osmolytes that act as

osmoprotectants in response to drought stress (Miao *et al.*, 2022). The earlier upregulation of genes linked to the galactose pathway in the sensitive genotype also correlates with the hypothesized more stressful state of this genotype, which may trigger the synthesis of galactose and related molecules to alleviate the impact of the studied mild drought.

Interestingly, genes linked to the ATP-dependent chromatin remodelling pathway showed downregulation from the 4th day of experiment in the sensitive genotype, while this downregulation was not present in the tolerant genotype until the last drought measurement. The downregulation of genes related to this pathway may be connected to stress memory. A plausible link between the observed downregulation of ATP-dependent chromatin-remodelling genes and drought stress memory is that plants prioritize survival and ATP economy over growth. Because chromatin remodellers control nucleosome positioning and histone dynamics, their reduced activity may promote the persistence of permissive states in ABA or stress-responsive genes while continuing to repress growth. This would lower the activation threshold and enable faster/stronger reinduction upon reexposure, a hallmark of epigenetic memory (Loka *et al.*, 2019; Lechowicz *et al.*, 2020; Miao *et al.*, 2022).

Rewatering after 5 days of mild drought changed the transcriptomic profile of both genotypes by enriching pathways not previously observed. Additionally, both genotypes seem to be able to revert some processes activated in response to mild drought after rewatering them by changing the regulation of genes reported in previous experimental sampling points. This is exemplified by the downregulation of genes related to the autophagy and starch and sucrose metabolisms pathways after rewatering in the sensitive genotype, and by the upregulation of genes linked to pathways related to the synthesis of nucleotides and fatty acids in both genotypes.

Sucrose is a carbohydrate that can act as osmoprotectant under drought stress, similarly to the role of fructans, which can also delay wilting by sustaining turgor, and help plants to maintain root activity under water deficit (Zwicke *et al.*, 2015; Loka *et al.*, 2019; Perlikowski *et al.*, 2020). Sucrose can be produced by degrading starch, which is commonly used by plants as a carbon storage source (Perlikowski, Czyżniejewski, *et al.*, 2016; Smith and Zeeman, 2020). In turn, starch can be utilized under reduced photosynthetic activity, such as that caused by stomatal closure due to drought stress, to sustain proper metabolism and cellular processes in early stages of drought stress (Thalmann and Santelia, 2017; AbdElgawad *et al.*, 2020). The sensitive genotype may activate starch degradation in response to water deficit, aiming to counteract the reduced availability of carbon sources due to stomatal closure and to synthesize osmoprotectants to mitigate drought stress damage. This pathway is activated in the 4th day of experiment in the sensitive genotype, while it is not active in the tolerant genotype until the end of the last day of mild drought. This pattern once more suggests that the sensitive genotype has a stronger and earlier response to the studied water deficit conditions than the tolerant genotype. While mild drought seems to affect more the sensitive genotype, this period of stress did not produce critical damage, since this genotype was able to reverse metabolic responses aimed to ameliorate the effect of mild drought when rewatered.

The synthesis of fatty acids is linked to the reparation of cellular membranes that may have been damaged by drought stress (Perlikowski, Kierszniowska, *et al.*, 2016). An activation of this and the nucleotides synthesis pathways can also be linked to growth, since lipids are needed for membrane biogenesis and nucleotides and lipids are necessary for cell division (Bonaventure *et al.*, 2003; Busche *et al.*, 2021). Their

upregulation after rewatering implies the reactivation of growth-related pathways in both genotypes. A similar phenomenon has been described in *L. perenne* and closely related species like *Festuca* spp. and in *Festulolium* hybrids (resulting from crossing *Festuca* x *Lolium*) when rewatering plants after periods of drought (Perlikowski, Czyżniejewski, *et al.*, 2016; Perlikowski, Kierszniowska, *et al.*, 2016; Ding *et al.*, 2023). Interestingly, Ding and colleagues reported that when comparing the number of tillers of perennial ryegrass plants that had been subjected to drought and rewatering periods versus control plants kept under normal watering conditions through the same number of days, the plants that were rewatered twice presented an increase in the number of tillers. The researchers associated this increase in tiller abundance to the upregulation of photosynthesis related genes, and proposed that a tight control of drought and rewatering cycles can be used to improve biomass accumulation and to reduce artificial water usage (Ding *et al.*, 2024). In our case, we also observed an upregulation of genes linked to photosynthesis pathways in both genotypes under drought stress conditions. This pathway was upregulated in the sensitive genotype only on day 3, but it presented upregulation on days 3 and 5 in the tolerant genotype. This pattern can lead to different hypothesis regarding the response to stress of the studied genotypes. Possibly, while both genotypes regulate their photosynthetic activity when sensing the stress, the sensitive one does it earlier than the tolerant genotype and in a negative manner. This might explain why the tolerant genotype presents upregulation of this pathway at two different sampling points, indicating that upon initially sensing drought, it can adjust its photosynthetic activity to continue functioning under water deficit, whereas prolonged lack of watering may trigger the same negative response presumed in the sensitive genotype. This correlates with the fact that the tolerant genotype presented upregulation of a pathway related to antenna proteins from the 3rd day onwards, suggesting that perhaps this genotype compensates stomatal closing by promoting the synthesis of more antenna proteins to maintain optimal photosynthesis levels. Further tests should be performed in parallel, including gas exchange studies and analysis of tillers protein profile, to elucidate if such acclimation response to drought stress is a mechanism used by the tolerant genotype.

The comparison of the transcriptomic data between the sensitive and tolerant genotype under mild drought also allowed us to identify genes that may be responsible for the dissimilar responses to the stress. Notably, genes previously reported to be involved in drought responses in other plants presented differential expression patterns between the studied genotypes. For example, a transcript associated with gene *TSO1*, which promotes root growth (Wang *et al.*, 2018), was upregulated in the last day of experiment in the tolerant genotype but downregulated in the sensitive. This suggests that the sensitive genotype responds to the studied mild drought earlier by suppressing root growth, while the tolerant induces root proliferation at later stages, similarly to what has been reported in barley drought tolerant plants (Janiak *et al.*, 2019). Under conditions of soil water deficit, many cool-season grasses adapt their root system architecture by increasing root depth, length, and biomass. This allows them to access residual moisture at deeper levels, enhancing their ability to avoid dehydration and sustain function throughout drought periods (Huang, DaCosta and Jiang, 2014; Zwicke *et al.*, 2015). Another stress related gene, *DHN3*, was also differentially expressed in the sensitive genotype, where it presented upregulation under drought conditions. Dehydrins are a family of proteins, belonging to group 2 of LEA proteins, induced by ABA and MAPK cascades that have important roles in response to dehydration and other

stresses (Hanin *et al.*, 2011) (Publication III). *DHN3* is a dehydrin gene that has been reported to be upregulated by water deficit in barley drought tolerant plants (Guo *et al.*, 2009). This was also the case in our study, where the expression of *DHN3* was upregulated in the tolerant genotype, but at lower levels and at a later stage than the observed for the sensitive one. The higher expression of *DHN3* in the sensitive genotype may reflect a more aggravated stressful state, together with an earlier and stronger response to drought of this genotype. While higher expression of dehydrins genes is normally linked to improved stress tolerance, the lower expression observed in the tolerant genotype may suggest that, in our study, the higher expression in the sensitive genotype indicates greater impact by the assessed mild drought. In concordance, *DREB2B*, a gene associated with drought tolerance when overexpressed in rice and Arabidopsis (Chen *et al.*, 2008; Matsukura *et al.*, 2010), was highly expressed in the sensitive genotype. The lack of upregulation of *DREB2B* in the tolerant genotype, further supports the hypothesis of a more stressed state of the sensitive genotype leading to a stronger and earlier response to drought.

Furthermore, *WSD1*, a gene linked to wax synthesis and shown to enhance drought tolerance in Arabidopsis when overexpressed (Abdullah *et al.*, 2021), was upregulated at two different sampling points in the tolerant genotype. These points coincide with the lowest RWC values recorded in this genotype, potentially relating to the role of *WSD1* in wax synthesis under drought conditions. Taken together, the low RWC measurements and the upregulation of *WSD1*, may reflect the promotion of cuticular wax synthesis in the tolerant genotype to maintain the water content in their leaves, a common response in plants when sensing periods of drought stress (Huang, DaCosta and Jiang, 2014; Loka *et al.*, 2019). Finally, a gene that has been previously proposed to be a negative regulator of drought stress response, *PAT1*, presented high levels of expression in the studied sensitive genotype. In Arabidopsis, *pat1* knockout mutants showed enhanced tolerance to drought, possibly linked to the participation of *PAT1* in the degradation of proteins resultant from ABA-dependent drought responses (Zuo *et al.*, 2021). Thus, elevated expression of this transcript, such as in the studied sensitive genotype, may be detrimental for plants under water deficit conditions. More details regarding the expression patterns of each gene can be found in section “4.3” and in “Table 1” of Publication I.

The overall expression trends of these genes align with the KEGG and physiological measurements results previously discussed, pointing towards the tolerant genotype having a delayed response towards mild drought, whereas the sensitive genotype senses the stress earlier and activates mechanisms aimed to reduce the impact of water deficit such as suppressing growth.

To further examine the possible mechanisms underlaying the different response towards mild drought of both phenotypes, co-expression analysis was used to determine if groups of DEGs were similarly expressed under drought conditions. This type of analysis is commonly used to identify clusters of genes that exhibit similar expression profiles across multiple samples and conditions, since genes with coordinated expression are likely to be co-regulated and may be involved in common biological pathways (L. Li *et al.*, 2023; Chen *et al.*, 2025). In our study, performing Weighted Gene Co-Expression Network Analysis (WGCNA) resulted in genes related to stress response, including genes linked to protein degradation, appearing as downregulated in the tolerant genotype but upregulated in the sensitive. On the contrary, the tolerant genotype showed no change in the expression of genes associated with cell division and growth, while the sensitive genotype presented downregulation of those same genes. Those modules, whose

expression pattern was statistically significantly different between the studied genotypes, were further analysed. From these subsets of groups, those whose GO was linked to growth were used to identify genes with high interactivity that were therefore considered as "hub genes" of the co-expressed transcripts. Similarly, Ding and colleagues (Ding *et al.*, 2024) used co-expression analysis to identify several hub genes involved in the promotion of tiller growth in plants exposed to drought and rewatering cycles. In our study, the growth-related pathways that were enriched in the hub genes belonged to microtubule activity, cell cycle regulation, cell division, cell wall biogenesis and DNA replication. Between the two genotypes, sensitive and tolerant, six growth-related modules showed statistically significant differences across the studied sampling points; and a hub gene was identified for each of these groups. The hub genes included a transcript involved in microtubule activity (*TUBB1*), genes linked to the synthesis of cell walls (*XOAT6* and *CSLA1*), a gene connected to shoot apical meristem development (*CDKB2-1*), and genes related to cell growth processes (*RHD3* and *TRS130*). More details regarding the expression patterns of each gene can be found in section "4.4" of Publication I. In summary, hub genes positively linked to growth were downregulated in the sensitive genotype under mild drought, whereas their expression levels remained relatively unchanged in the tolerant. In some cases, downregulation of these genes was present on both genotypes, but it appeared earlier in the sensitive one and in some cases stabilized and returned to almost basal values in the tolerant genotype. Altogether, these findings indicate that cell growth and cell wall formation are more affected in the sensitive genotype, and that the tolerant one can preserve growth under the studied mild drought conditions by stabilizing the expression of the identified hub genes.

4.3 Impacts of freezing temperatures on electrolyte leakage (Publication II)

Subzero temperatures (< 0 °C) can damage the membrane of cells and cellular compartments, leading to the release of certain molecules or leakage (Thomashow, 1999; Rapacz *et al.*, 2014). By quantifying this phenomenon after exposing plant tissues to freezing temperatures, the tolerance towards this kind of stress can be determined (Winfield *et al.*, 2010; Ergon, 2017). In our study, electrolyte leakage (EL) was used to evaluate how different genotypes responded to freezing temperatures after a period of cold acclimation. This assay relies on the conductivity-based quantification of injuries produced by temperatures below zero and is commonly used in grasses to estimate the temperature at which plants are damaged by said temperatures (e.g., LT50) (Winfield *et al.*, 2010; Ergon, 2017). In our work, the electrolyte leakage was used to determine the tolerance of the evaluated genotypes towards two specific temperatures, -12 °C and -14 °C. For this, freezing tolerance was defined as indirectly proportional to the percentage of EL measured at the target temperatures, where higher levels of EL correspond to lower levels of tolerance. Two representative genotypes with low EL values were selected as tolerant based on this assessment, while two other genotypes with high EL values were identified as sensitive and used in subsequent experiments. This classification was further confirmed by the principal component analysis, where the sensitive genotypes were clustered separately from the tolerant ones throughout the different timepoints (Fig. 3B, Publication II).

4.4 Transcriptomic profile under cold acclimation (Publication II and Publication III)

To evaluate the effect of cold acclimation in the studied genotypes, three different timepoints, corresponding to early, mid and end of acclimation, were compared against samples obtained after twenty-one days of establishing the plants at stable temperature conditions (control conditions, T1). Cold acclimation is an important process that allows perennial cool-season grasses to survive winter conditions and to resume growth when temperatures rise (Rapacz *et al.*, 2014; Ergon, 2017) (Publication III). Differential gene expression analysis in our study revealed that the sensitive genotypes exhibited a lower number of DEGs (11125) compared to the tolerant ones (12937) when contrasting control and treatment conditions. From these DEGs, a majority were shared between the sensitive and tolerant genotypes (7802), but the tolerant genotypes presented a higher number of genotype specific DEGs (5135) than the sensitive ones (3323). In the contrast between genotypes during cold acclimation, the tolerant genotypes had a higher number of up and downregulated DEGs compared to the sensitive genotypes (Publication II, Fig. 4B).

In the Baltic region, warmer winters have been accompanied by a decrease of winter damage in forage crops (Kemešytė, Jaškūnė and Statkevičiūtė, 2020; Kemešytė *et al.*, 2023). Still, freezing tolerance is a limiting factor influencing the ability to cultivate perennial ryegrass in this region (Publication III). To understand how cold acclimation can influence this tolerance, the DEGs of sensitive and tolerant genotypes were compared against their basal conditions and between the different genotypes, revealing that the tolerant ones presented a higher number of DEGs during cold acclimation than the sensitive genotypes. The lower number of DEGs in the sensitive genotypes suggests that they may have either an impaired response towards low temperature stimuli or that they are unable to undergo cold acclimation. Both could be responsible for the lower tolerance towards freezing, since perennial plants undergo cold acclimation to prepare for winter and its below zero temperatures (Publication III). Without prior acclimation, plants may experience increased stress from cold exposure, hindering their capacity to resume normal functions in spring and potentially leading to death, depending on the severity of freezing damage (Thomashow, 1999; Kurepin *et al.*, 2013) (Publication III).

At the beginning of this acclimation process (T2), KEGG pathway analysis showed that both genotypes upregulated genes involved in the MAPK signalling pathway, which is connected to signalling mechanisms involving calcium in response to stress and is linked to cold acclimation in different plant species (Guo, Liu and Chong, 2018; Jagodzik *et al.*, 2018). In the tolerant genotypes, genes related to the circadian rhythm were enriched during the cold acclimation process. Similarly, previous studies showed the possible role of circadian rhythm related genes in regulating cold-responsive genes such as *DREB1* (Jang *et al.*, 2024). The sensitive genotypes showed upregulation of genes connected to the proline and arginine metabolism, two molecules that are known to act as osmoprotectants and membrane stabilizers in response to cold stress (Winfield *et al.*, 2010; Rapacz *et al.*, 2014). Additionally, proline has been shown to accumulate during cold acclimation and to be able to serve as an energy source under stress conditions (Liang *et al.*, 2013). This upregulation was not present in the tolerant genotypes, which opposes previous observations in relation to cold responses in *L. perenne*. Previous reports indicate a higher accumulation of proline in the crown region and leaves of freezing tolerant perennial ryegrass plants following cold acclimation compared to

non-tolerant plants (Hoffman *et al.*, 2010; Bocian *et al.*, 2015). This lack of upregulation of proline-related pathways during the studied cold acclimation suggests that these tolerant perennial ryegrass genotypes may employ alternative winter hardiness mechanisms not previously reported.

In the late acclimation timepoint (T4), the tolerant genotypes showed upregulation of genes related to the inositol phosphate metabolism, similarly to what has been previously seen in *Brassica napus* in response to cold. In this species, inositol seems to improve cold tolerance by inducing the flux of calcium ions under low temperature conditions. Additionally, the researchers reported that inositol seemed to inhibit the expression of a gene, *BnCBL1-2*, whose overexpression led to impaired cold responses in *B. napus* (Yan *et al.*, 2022). In grasses, inositol can act as precursor of osmolytes that accumulate in response to cold, such as those belonging to the raffinose family, and as a signalling molecule involved in the activation of the ICE-CBF-COR pathway under cold stress (Thomashow, 1999; Rapacz *et al.*, 2014) (Publication III). The upregulation of this pathway at the end of the acclimation period, suggests that the tolerant genotype may be accumulating derivatives of inositol in response to cold. The subsequent upregulation of the same pathway under freezing temperatures, complements this theory of a higher accumulation of osmoprotectants. Additionally, this upregulation could hint towards the inositol linked activation of the ICE-CBF-COR pathway in the tolerant genotypes at the end of the cold acclimation period and under freezing stress, but not in the sensitive genotypes. Therefore, the differences in inositol metabolism and inositol related signalling pathways may be linked to the dissimilar tolerance towards low temperature of the studied genotypes.

Genes related to fatty acid elongation were also enriched during cold acclimation in both genotypes. The upregulation of these genes opposes what has been previously reported in maize, where the roots of plants exposed to cold stress showed upregulation of genes involved in fatty acid elongation and synthesis (Guo, Liu and Chong, 2018). Similarly, tolerant genotypes also presented downregulation of genes linked to glutathione metabolism in the cold acclimation samples. Glutathione can act as an antioxidant that mitigates the damage produced by the accumulation of ROS molecules under cold stress, and it can also modify the activity of proteins involved in cold acclimation processes (Dorion, Ouellet and Rivoal, 2021). In wheat, plants with increased tolerance to cold stress presented accumulation of glutathione and glutathione derived enzymes after a period of cold acclimation, hinting towards a direct relation between higher levels of glutathione and improved cold tolerance (Lu *et al.*, 2023).

The differences in enriched pathways observed in our results were sometimes contrary to what has been observed in other grasses, such as the downregulation of DEGs associated with fatty acid elongation and glutathione metabolism. This differential expression patterns suggest that the evaluated perennial ryegrass genotypes may exhibit distinct responses during cold acclimation compared to previous observations in other grasses like wheat and maize.

4.5 Differential gene expression and transcriptomic profile under freezing conditions (Publication II)

To assess the effect of freezing temperatures, the samples obtained after exposing the plants to two different subzero temperatures (-5 °C and -10 °C) were contrasted against control and cold acclimation conditions. Our study revealed that the tolerant genotypes

had a higher number of up and downregulated DEGs than the sensitive ones when comparing the early freezing stage (T5) and the control conditions (T1). In the second freezing timepoint (T6), the sensitive and tolerant genotypes had a similar number of up and downregulated DEGs, while these patterns changed when comparing the end of acclimation (T4) against the samplings from the two tested freezing conditions. In both cases, the sensitive genotypes showed a higher number of upregulated DEGs, while the tolerant genotypes presented a higher number of downregulated DEGs in the first freezing timepoint but a lower number of DEGs than the sensitive genotypes in the second one. Moreover, whereas the number of DEGs remained similar among the samples belonging to the freezing conditions in the tolerant genotypes, in the sensitive the number of up and downregulated DEGs increased when the temperature lowered (Publication II, Fig. 4B).

Performing KEGG pathway analysis showed that both genotypes presented similar responses to freezing stress, such as the upregulation of genes related to carbon, sucrose, and starch. During cold acclimation and freezing, starch is remobilised to other sugars (like sucrose and fructans), which serve as osmoprotectants that stabilise membranes and help mitigate oxidative damage, while also providing energy when photosynthesis is impaired (Rapacz *et al.*, 2014; Ergon, 2017) (Publication III). Similarly, although both genotypes exhibited upregulation of genes associated with galactose metabolism, this positive regulation was only observed in the tolerant genotypes at the final freezing timepoint. Galactose, as described in drought stress, acts as a source for the synthesis of osmoprotectants in response to freezing temperatures. Additionally, galactose is also part of the galactolipids found in thylakoid membranes. Freezing stress induces a change in the ratio and composition of these galactolipids, aiming to reduce freezing damage to chloroplasts (Rapacz *et al.*, 2014). Therefore, galactose has an important role in preventing and ameliorating oxidative stress and membrane damage, which can ultimately lead to electrolyte leakage (Nishizawa, Yabuta and Shigeoka, 2008; Moellering, Muthan and Benning, 2010; Vu *et al.*, 2022). The earlier upregulation of genes related to galactose metabolism in the sensitive genotypes may indicate a stronger impact of cold in these plants, explaining why the galactose pathway was positively enriched in the sensitive genotypes already in the mid-acclimation timepoint (T3). Perhaps the temperatures used to acclimate the genotypes were low enough to induce damage into the sensitive genotypes, while the same damage may not be present in the tolerant ones until the temperature further decreases. This more abrupt effect may explain why the EL was significantly lower in the sensitive genotypes, reaching levels above 20 % under -12 °C, but did not go beyond this percentage in the tolerant genotypes until reaching lower temperatures (Fig. 3A, Publication II). Genes linked to pathways related to cell division, such as the DNA replication and the ATP-dependent chromatin remodelling pathways, were downregulated in both genotypes under freezing conditions, suggesting that growth may be negatively affected by subzero temperatures. This effect appeared to be more severe and quicker in the sensitive genotypes, since genes related to the DNA replication pathway were downregulated earlier and in conjunction with other cell division and growth pathways not enriched in the tolerant genotypes, such as the motor proteins and homologous recombination pathways. Furthermore, ribosome related genes were downregulated in the tolerant genotypes in the freezing timepoints but not in the sensitive ones. A decrease in translational activity has been linked with a shift in resources utilization and energy preservation, aiming to mitigate oxidative damage and reduce growth (Rapacz *et al.*, 2014; Ergon, 2017). Thus,

the downregulation of genes linked to the ribosome pathway may further indicate a suppression of growth in the tolerant genotypes, together with a translational reprogramming, in response to freezing conditions not observed in the sensitive genotypes. This difference may result from an impaired cold acclimation process in the sensitive genotypes, which prevents them from effectively changing the synthesis of proteins towards a stress related metabolism.

4.6 Genes with differential expression in sensitive and tolerant genotypes (Publication II)

Several genes were differentially expressed between the sensitive and tolerant genotypes after performing a comparative transcriptomic analysis. Genes that have been previously linked with responses towards stress or freezing conditions were further analysed, aiming to discover which of said genes may be responsible for the different tolerance to freezing in the studied genotypes. More information regarding these genes, including the timepoints in which they are differentially expressed, can be found in "Table 1" and on the last section of the Discussion of Publication II.

Genes, known to play roles in the ICE-CBF-COR signalling pathway and involved in molecular responses towards stress, were highly expressed in the tolerant genotypes. These set of genes coded for proteins including ICE2, DHN3-like, heat and cold shock proteins such as HSF A2a-like and CS120-like, among others. Several of these proteins protect cellular structures, like membranes, under stressful conditions (Karlson and Imai, 2003; Hundertmark and Hincha, 2008; Fursova, Pogorelko and Tarasov, 2009; Hanin *et al.*, 2011). Consequently, their elevated expression in the tolerant genotypes may explain the significantly lower levels of EL observed in these genotypes compared to the sensitive ones under the studied subzero temperatures. The tolerant genotypes appear to maintain better cellular integrity under freezing conditions, thus enhancing their resilience to this stress and reducing the leakage of ions and other molecules into the extracellular space. EARLI1 is another protein related to the preservation of membranes stability under low temperature conditions (Wilkosz and Schläppi, 2000). In our study, two different transcripts were identified as *EARLI1*, one with higher expression in the tolerant genotypes and the other with higher expression in the sensitive ones. This latter transcript presented very low expression in the tolerant genotypes, while it constantly increased its expression levels with the progression of the experiment in the sensitive genotypes. In the case of the *EARLI1* transcript with differentially higher expression in the tolerant genotypes, the sensitive genotypes presented a similar expression pattern but with lower levels than the tolerant counterparts. Together, this could indicate that the studied genotypes present two paralogs of *EARLI1*, the expression of which differs in both sets of genotypes. The expression level of an *EARLI1* paralog, which is highly expressed in sensitive genotypes, may negatively impact the expression of the paralog that is differentially more expressed in tolerant genotypes. In turn, this negative impact may hinder the ability of the sensitive genotypes to preserve the integrity of their cellular membranes, which could explain their significantly higher levels of EL under freezing conditions (Fig. 3A, Publication II). Likewise, two transcripts with different expression patterns between the sensitive and tolerant genotypes were identified as being translated into fructosyltransferase 1-SST. This protein is involved in the synthesis of fructans and has been previously connected to cold tolerance in perennial ryegrass (Abeynayake, Byrne, *et al.*, 2015; Abeynayake, Etzerodt, *et al.*, 2015). Both transcripts

exhibited similar expression patterns, peaking during cold acclimation and decreasing after freezing exposure. In wheat plants tolerant to freezing, the expression of *1-SST* was shown to be downregulated under subzero temperatures; suggesting that the studied genotypes may behave in a similar manner to wheat plants. In addition, the presence of two paralogs of *1-SST* with different expression patterns in the studied genotypes, indicate that the one with higher expression in the tolerant genotypes may have a stronger impact in the synthesis of fructans needed for preventing osmotic and oxidative damage under freezing conditions. Once more, this differential expression may be the reason why the sensitive genotypes show higher levels of EL when exposed to below zero temperatures (Fig. 3A, Publication II).

Genes coding for lipoxygenases and proteins like DREB1 were more expressed in the sensitive genotypes. Lipid oxidation is a signalling mechanism that can lead to damage to cellular membranes when it exceeds the antioxidant and repair capacity of cells (Niki *et al.*, 1991; Alché, 2019). The activity of lipoxygenases can lead to lipid peroxidation when overexpressed (Lim *et al.*, 2015), hence connecting the higher EL levels of the sensitive genotypes with possible damage to their cellular membranes caused by the increased activity of lipoxygenases observed in the transcriptomic analysis under freezing conditions. The MYC2 transcription factor is known to be a positive regulator of the ICE-CBF-COR signalling pathway (Song *et al.*, 2022). Both genotypes seemed to upregulate *MYC2* at the beginning of cold acclimation, but the tolerant genotypes showed an elevated expression compared to the sensitive ones. The expression levels of *MYC2* remained higher through the evaluated temperatures, reaching a peak of upregulation with the onset of freezing in the case of the tolerant genotypes. Dissimilar expression levels may reflect a better acclimation process in the tolerant genotypes, that allowed them to cope better with freezing conditions. On the contrary, the fact that the expression levels of *MYC2* after freezing conditions remained similar to those observed at the beginning of the acclimation period in the sensitive genotypes, hints towards the impaired ability of these genotypes to prepare for subzero temperatures. In Arabidopsis, the overexpression of *MYC2* from wheat (*TaMYC2*) improved the tolerance of plants towards freezing temperatures, showing that this could be a suitable target for improving freezing tolerance in cereals (R. Wang *et al.*, 2022). Another transcript associated with the ICE-CBF-COR signalling pathway, *COR143*, was also shown to be differentially expressed in our analysis. *COR143* is linked to cold stress response, presenting upregulated expression in Arabidopsis plants (Breton *et al.*, 2003; Hu *et al.*, 2021; Hwarari *et al.*, 2022). In our transcriptomic analysis, *COR143* had similar expression patterns in both genotypes, but it was differentially upregulated during cold acclimation in the tolerant genotypes. This finding supports the hypothesis that the sensitive genotypes exhibit a compromised or less efficient cold acclimation process. Moreover, a transcript identified as *GRP1* was also differentially highly expressed in the tolerant genotypes throughout the different evaluated timepoints, correlating with previous findings. *GRP1* has been suggested to be involved in improved tolerance towards subzero temperatures after cold acclimation in perennial ryegrass (Shinozuka *et al.*, 2006). In concordance, transgenic Arabidopsis plants encoding a rice homologue of a *GRP* gene also presented enhanced freezing tolerance after undergoing cold acclimation (Kim *et al.*, 2010). Finally, a transcript associated to the negative regulator of cold response *CRPK1* was differentially expressed in the sensitive genotypes during freezing conditions. CRPK1 is a protein involved in the degradation of CBF proteins, hinting towards a negative regulation of freezing tolerance (Liu *et al.*, 2017). In our analysis, the expression levels of

CRPK1 remained relatively stable throughout the different timepoints in the sensitive genotypes, while in the tolerant ones the expression of *CRPK1* seemed to be progressively downregulated after cold acclimation. This dissimilar expression pattern of a negative regulator of freezing tolerance supports the hypothesis that the sensitive genotypes present an inadequate cold acclimation process, making them unable to inhibit the degradation of CBF proteins which in turn decreases their ability to cope with freezing temperatures. Furthermore, the fact that in the tolerant genotype the expression of *CRPK1* is downregulated after cold acclimation, suggest that this could be a suitable target to improve the tolerance of ryegrass towards subzero conditions.

4.7 Protoplasts isolation and transformation (Publication IV)

Two different methodologies were compared for the isolation of perennial ryegrass protoplasts: cutting the tillers or seedlings with a razor blade (also referred to as the classical method in this publication) or disintegrating the plant material with the help of a blender. For both methods, different conditions were tested to determine the most suitable settings to obtain a high number of viable or alive cells that could be further used for transformation purposes. The tested conditions included the cellulase content of the enzymatic solution used to degrade the plant cell wall, variable enzymatic treatment lengths, the use of different mannitol concentrations for a pretreatment aiming to induce plasmolysis of the cells, and the application of vacuum to enhance the infiltration of the enzyme solution into the plant tissue. Additionally, for the blending methodology, four different setups using a different number of "pulses" were studied. To favour a proper comparison of both methodologies, the same amount of initial plant material was used, 2 g of fresh weight (FW) tillers or seedlings. Counting alive cells after each of the mentioned treatments was used to determine the yield of each condition. For this, fluorescein diacetate (FDA, 5mg/mL) was added to a suspension of washed protoplasts to detect fluorescence in viable or alive cells with the help of a fluorescence microscope.

When using a blender, applying five pulses to the plant material provided the statistically significant highest number of viable protoplasts (3.86×10^4 cells per gFW) in comparison to testing 1, 3 or 10 pulses (Figure 2, Publication IV).

Using a razor blade to process the plant material is one of the most common methods when aiming to isolate protoplasts. This step is generally followed by the degradation of plant cell walls using an enzymatic treatment. In turn, the enzymatic degradation of cell walls is often accompanied by gentle agitation. The enzymatic profile of the solution used for this purpose depends on the composition of the digested cell wall; thus, it varies among different plant species. Since cellulose is one of the main components of the cell wall of perennial ryegrass tillers and cellulase has been shown to play an important role in the degradation of mesophyll cell walls in *L. perenne*, different cellulase concentrations were tested in this study (Gordon *et al.*, 1985; Vetharaniam *et al.*, 2014). The rest of the enzymatic composition was based on previous protocols describing the isolation of perennial ryegrass protoplasts (Yu *et al.*, 2017; Davis *et al.*, 2020). Furthermore, previous published works presented very different enzymatic treatment lengths, 6 (Yu *et al.*, 2017) or 20 hours (Davis *et al.*, 2020). Based on this big variation, different incubation times with the enzymatic solution were also tested in our study, to find the shortest possible treatment providing the highest number of viable cells. We compared enzymatic solutions with four different cellulase concentrations (1.5, 2, 2.5, and 3%) tested during four different incubation times (8, 12, 16, and 20 h). The enzyme solution with 2% of

cellulase yielded the best results, in terms of alive protoplasts, in all the tested incubation times. Regarding the length of the enzymatic treatment, 8 hours of incubation provided the highest number of alive cells (Figure 3, Publication IV).

The parameters previously determined to provide the highest yield, enzyme solution with 2% cellulase and incubation of 8 hours, were used to test the mannitol pretreatment and vacuum conditions. The pretreatments of plant materials can be performed before or after said material has been processed, by for example cutting the leaves with a razor. These pretreatments aim to further improve the number of viable cells isolated, due to the ability of some solutes to induce the separation of cellular membranes from cell walls through a process known as plasmolysis (Reed and Bargmann, 2021). From the four different mannitol concentrations (0.2, 0.3, 0.5, and 0.6 M) used for the plasmolysis of the plant material, 0.5 M provided significantly higher number of alive protoplasts than the rest of concentrations (Figure 4A, Publication IV). When comparing the use of vacuum to facilitate the infiltration of the enzyme solution into the plant material against no vacuum treatment, a statistically significant higher number of viable cells was observed when applying 71 kPa of vacuum pressure. In addition, a sucrose cushion was used to increase the number of alive cells together with reducing cellular debris. This aimed to improve transformation efficiency and downstream applications, which can be hindered by a high number of dead protoplasts and excess of debris (Chen *et al.*, 2023). Similar gradient-based separations of viable protoplasts had not been previously reported in the isolation of perennial ryegrass protoplasts.

The transformation of protoplasts was done using a solution containing polyethylene glycol (PEG) and calcium ions to favour the delivery of binary vectors, similar to what has been previously described by other authors transforming *L. perenne* protoplasts. Three different types of plasmids were tested, one containing individual guide RNAs (gRNAs) and an EGFP cassette, another one encoding five different gRNAs and a ZsGreen cassette, and a third plasmid with six gRNAs, a ZsGreen cassette and an intronized *Zea mays* codon optimized Cas9 (Materials and Methods, Publication IV). EGFP and ZsGreen are two fluorescent proteins that were used to assess the transformation efficiency by quantifying the number of cells presenting fluorescence 48 hours after transformation. Two different genes, *CBP20* and *CRPK1*, were targeted in this research. *CBP20* is associated with cuticular wax synthesis, and knockout mutants in barley showed improved drought tolerance (Daszkowska-Golec *et al.*, 2017, 2020). *CRPK1* is a negative regulator of cold response, that was identified as differentially expressed in a transcriptomic analysis of frost-sensitive *L. perenne* plants (Liu *et al.*, 2017; Pashapu *et al.*, 2024). For the *LpCBP20* gene, two different types of vectors were used, encoding either a single gRNA or five guides. For the *LpCRPK1* gene, a vector containing an intronized Cas9 was used, encoding six different gRNAs. The transformation using each type of plasmid was accompanied by transforming cells with a control plasmid not encoding gRNAs.

In the case of the plasmid encoding single gRNAs, two variations of the vector presenting two different gRNAs were used. No statistically significant differences were observed between the control plasmid (pEGFP) and the vectors encoding gRNAs (p195 and p229) (Figure 5A, Publication IV). Similarly, the vector with five gRNAs (pCBP20_5g) showed no statistically significant higher number of transformed cells than the control plasmid (pDelta) (Figure 5B, Publication IV). Since ZsGreen is intrinsically brighter than EGFP (Susič, Bohanec and Murovec, 2014; Cho *et al.*, 2019), the two types of plasmids targeting *LpCBP20* cannot be compared due to encoding different fluorescent markers.

Additionally, the expression levels of EGFP may be lower than expected in the pHSE401/EGFP plasmids, since two other transcriptional units are controlled by the same promoter (CaMV 35S). In binary vector systems, transcriptional silencing can reduce the expression of genes controlled by the same promoter, resulting in inconsistent gene expression across different cassettes (Altpeter *et al.*, 2016; Anjanappa and Gruissem, 2021). Hence, transcriptional silencing may interfere and reduce the expression of EGFP in these plasmids, leading to a lower estimation of transformation.

For the vectors presenting an intronized Cas9, both the control (pCtrl_iCas9) and gRNAs (piCas9_CRPK1) encoding plasmids had higher transformation efficiencies than those observed from the other types of plasmids, with more than 40% of the visualized protoplasts presenting fluorescence. Moreover, no statistically significant differences in terms of transformation efficiency were present between the control plasmid (pCtrl_iCas9) and the plasmid encoding gRNAs (piCas9_CRPK1) (Figure 5C, Publication IV). The higher transformation efficiency observed in the plasmids targeting *LpCRPK1* is probably not due to transcriptional silencing, since the same cassette expressing ZsGreen is present in these vectors and in the plasmid with five gRNAs targeting *LpCBP20*. The increased number of quantified fluorescent cells may be related with the experience in performing the transformation itself. While the transformations with the vectors targeting *LpCBP20* were performed in parallel, the transformation with the plasmids aiming to edit *LpCRPK1* were done at a later stage.

4.8 Evaluation of editing efficiency (Publication IV)

A previous assay by Zhang and colleagues briefly mentioned the use of perennial ryegrass protoplasts for testing gRNAs before their use in transforming other *L. perenne* plant material. But no information regarding the editing efficiency of the protoplasts was provided (Yunwei Zhang *et al.*, 2020). In our work, two days after the transformation with binary vectors, genomic DNA from the protoplasts was extracted. Different amplicons were generated by PCR depending on which vectors were used to transform the protoplasts. These DNA regions were then Sanger-sequenced and checked for the presence of indels using decomposition-based analysis (TIDE tool), that evaluates the editing efficiency based on the percentage of indels present in a sample (Brinkman *et al.*, 2014). Following TIDE's recommendations, only those samples presenting a R^2 value equal to or greater than 0.9 and whose indel frequencies had a p value below 0.001 were considered for this study.

Since two types of vectors were used to target the same exon of *LpCBP20*, the editing efficiency of the shared gRNAs was compared. In the first type of vectors, encoding a single gRNA, the average editing efficiency was not statistically significant but appeared to be lower than that observed for the plasmid encoding five guides (7.8 vs 8.6%, respectively) (Figure 6A and B, Publication IV). When comparing the efficiency of the same guides in the two types of plasmids, g196 showed similar indel frequencies in both types of vectors (~ 8%), while g229 had a higher editing efficiency in the multiplex plasmid than in the single encoding vector (9% vs 7% indels, respectively) (Figure 6A and B, Publication IV). Since both guides were targeting the second exon of *LpCBP20*, it is possible that a cumulative effect may be responsible for the higher editing efficiency observed for g229 in the multiplex plasmid. According to literature, using multiple guides that target the same genomic region can increase the chances of inducing frameshifts that generate knockouts, as DSBs in close proximity can induce larger deletions or insertions during NHEJ-mediated repair (Chen *et al.*, 2019; Wada *et al.*, 2020). On the

other hand, multiplexing can be accompanied by a reduction of the editing efficiency of the different guides due to competition for the same pool of nucleases (Chen *et al.*, 2019; Laforest and Nadakuduti, 2022). In our study, the same amplified region was used to examine the presence of indels generated by different gRNAs targeting the same exon. This may explain the higher indel frequency observed with multiplex guides, as TIDE analyses only one guide's output at a time. Other decomposition-based programs provide the option to simultaneously analyse the output of more than one guide, but were not considered due to not being the best choice when expecting low editing efficiencies (below 10%), (Brockman *et al.*, 2023; Aoki *et al.*, 2024). This was the case for most of our transformation experiments.

In addition, the use of a codon optimized Cas nuclease has also been documented to increase editing efficiency in plants (Hussain, Lucas and Budak, 2018). This could also explain why guide 229 had a higher editing efficiency in the multiplex plasmid, since this vector encodes a *Triticum aestivum* codon optimized Cas9 (TaCas9), while the vector with a single gRNA presents a *Zea mays* codon optimized Cas9 nuclease (ZmCas9). Both nucleases have been optimized to present higher expression levels in monocots, but wheat is a closer relative of *L. perenne* than maize. Perennial ryegrass and wheat belong to the same Poaceae subfamily, Pooideae, while maize belongs to a different one, Panicoideae (Soreng *et al.*, 2022).

Regarding the plasmid aiming to edit three different paralogs of *LpCRPK1*, two of the six gRNAs encoded in the plasmid did not generate results compliant with the cutoff of TIDE and were excluded from further analysis. Of the other four guides, which targeted the first two exons of paralogs 190 and 232, three (excluding guide 232-2) presented editing efficiencies above 10% (Figure 6C, Publication IV). No statistically significant differences were obtained when comparing the indels frequencies of the four different guides. Nonetheless, the average editing efficiency of this plasmid seemed to be higher than the observed for the other two types of binary vectors. The addition of introns has been shown to increase gene expression due to a phenomenon known as intron-mediated enhancement (IME). IME leads to an increment in the levels of mRNAs which can be accompanied by an improved translation and a decrease of transgene silencing that will in turn produce more of a specific protein such as Cas9 (Rose, 2004; Rose *et al.*, 2011). In plants, the addition of introns in the CDS of Cas9 nucleases has been proven to increase the editing efficiency of non-intronized nucleases by generating a higher number of mutations in the targeted genomic regions (Castel *et al.*, 2019; Grützner *et al.*, 2021). Moreover, when comparing intronized Cas9 nucleases, those with a higher number of introns in their CDS outperformed those with fewer introns (Lawrenson *et al.*, 2024). In our study, the Cas9 encoded in the binary vector targeting *LpCRPK1* presented thirteen introns in its CDS and has been previously shown to increase editing efficiency in barley, with over 90% of the analysed transformed plants presenting mutations (Lawrenson *et al.*, 2024).

5 Conclusions

The studies performed throughout this thesis aimed to identify genes involved in the differential tolerance towards drought and low temperature stresses in perennial ryegrass plants. Additionally, a platform that could be used to test genome editing agents targeting said genes was another outcome of this thesis, aspiring to facilitate future applications of genome editing in *L. perenne*.

The transcriptomic analysis of plants with different tolerance levels against drought stress revealed that the genotypes studied presented dissimilar molecular responses to water deficit. The sensitive genotype seemed to detect and respond to mild drought earlier than the tolerant one and appeared to be more affected by the studied stressful conditions, towards which it responded by stopping leave growth in advance. On the contrary, the tolerant genotype showed signs of stress only on later stages and responded to it by promoting root growth. Furthermore, the tolerant genotype was able to maintain leave growth for a longer period, and according to the results from the co-expression analysis, it may be capable of doing so by adapting its molecular responses to prolonged drought stress. Specific genes may be responsible for the poor performance of the sensitive genotype under the studied mild drought, such as *PAT1*. Other DEGs point towards an inherent adaptation of the tolerant genotype which enable it to maintain growth under the evaluated water deficit conditions, such as the possible promotion of cuticular wax synthesis and root proliferation mediated by the expression of *WSD1* and *TSO1*, respectively. The identification of these DEGs can be used to improve the tolerance of specific genotypes, by modifying their expression through genome editing techniques like CRISPR-Cas.

Studying the impacts of cold acclimation and freezing stress in plants with dissimilar tolerance towards the stress revealed that similar mechanisms were activated by both sets of genotypes. Even so, the sensitive genotypes showed to have fewer number of DEGs during the cold acclimation period in comparison to the tolerant genotypes. This suggests an impaired response to cold temperatures, which hinders the ability of the sensitive genotypes to prepare for freezing conditions due to not undergoing proper cold acclimation. This deficit then translates in higher susceptibility to subzero temperatures, as shown in their higher electrolyte leakage values. Additionally, this lack of readiness for below-zero temperatures may be further exacerbated in sensitive genotypes by their dissimilar modulation of *CRPK1*, a negative regulator of cold responses. Tolerant genotypes downregulated this gene after cold acclimation, whereas sensitive ones maintained relatively stable expression throughout the experiment. Additionally, the tolerant genotype upregulated some genes and pathways that were not enriched nor differentially expressed in the sensitive genotypes, (e.g., genes related to the inositol metabolism and signalling pathway) providing insight into which mechanisms may be responsible for the dissimilar tolerance towards freezing. Candidate genes that may be capable of enhancing the tolerance towards freezing in perennial ryegrass, like *MYC2* and *CRPK1*, were identified as dissimilarly expressed in the studied genotypes. Utilising NGTs such as CRISPR-Cas could enable the development of novel *L. perenne* lines that are better equipped to withstand freezing conditions by targeting these candidate genes.

An optimized method for the isolation of perennial ryegrass protoplasts was established. The number and overall state of the isolated cells was suitable for downstream applications, such as the evaluation of different editing agents. We demonstrated that PEG-mediated transformation of perennial ryegrass protoplasts with binary vectors is a

suitable method to evaluate the editing efficiency of said plasmids. By extracting genomic DNA from the pool of transformed cells, the presence of indels was analysed using a decomposition-based tool (TIDE) capable of detecting the editing efficiency and frequency of indels from Sanger-sequenced amplicons. The proposed technique is both fast and low cost in comparison with other methods like the evaluation of genome editing outputs through NGS. The possibility of testing transformation vectors *in vivo* can help improve the chances of generating a transformed plant, since the editing efficiency observed in protoplasts closely resembles the expected one when using different plant materials such as calli. In turn, this can speed up the timeline needed to generate plants with desirable traits through NGTs, such as organisms with improved tolerance to abiotic stress.

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References

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Abstract

Transcriptomic insights and testing of genome editing tools for climate-resilient perennial ryegrass

Lolium perenne (perennial ryegrass) is one of the most cultivated cool-season forage grasses in temperate regions, especially throughout Europe. Climate change is exacerbating temperature extremes and altering rainfall patterns, subjecting cool-season grasses to increasingly recurrent and intense low-temperature and drought spells. The exposure to adverse climate will translate into reduced growth and therefore biomass accumulation and forage yield.

This thesis aimed to identify mechanisms and genes involved in tolerance towards drought and cold stress through transcriptomics studies, with the goal of discovering candidate genes useful for future breeding programs. Furthermore, a platform based on protoplasts was developed to test editing efficiency *in vivo* before stable transformation of perennial ryegrass calli.

Plants with differing levels of drought tolerance were analysed at the transcriptomic level, revealing that the sensitive genotype was more affected by the mild drought treatment and responded earlier than the tolerant genotype. This genotype did not present the decrease in leaf growth observed in the sensitive one and promoted root development and synthesis of cuticular waxes in later stages of the studied stress conditions, as hinted by the upregulation of *TSO1* and *WSD1*. Additionally, specific genes such as *PAT1* presented expression patterns that suggested a role in the poor performance of the sensitive genotype under mild drought.

Similarly, cold acclimation and freezing stress were also evaluated through transcriptome analysis of genotypes with dissimilar responses to said conditions. A difference in the number of DEGs was observed, where the sensitive genotypes had a smaller number of DEGs during cold acclimation than the tolerant genotypes, implying a possible hindered ability to prepare for below-zero temperatures. This could be the reason why the sensitive genotypes presented a higher electrolyte leakage when exposed to subzero temperatures than the tolerant genotypes. Furthermore, a known negative regulator of cold response (*CRPK1*) was highly expressed in the sensitive genotypes under low temperature conditions, hinting once more towards an improper cold response of these genotypes.

The isolation of perennial ryegrass protoplasts was optimised to create a testing platform capable of screening gene-editing components *in vivo*. The analysis of the genomic DNA of protoplasts transformed with binary vectors using PEG provides a suitable technique to evaluate the presence of indels resulting from the activity of the nucleases guided by the guide RNAs, encoded in the vectors. Decomposition analysis using the tool TIDE can detect the editing efficiency and frequency of indels from Sanger sequenced amplicons. Compared to other methods, such as NGS-based evaluation of genome editing outcomes, the proposed technique is faster and more cost-effective. The *in vivo* testing of transformation vectors can help improve the chances of generating a transformed plant and shorten the time required to generate organisms with desirable traits, such as improved abiotic stress tolerance.

Lühikokkuvõte

Transkriptoomi põhised teadmised ja genoomi täppismuutmise tööriistade testimine kliimakindla karjamaa raiheina jaoks

Lolium perenne (karjamaa raihein) on jahedates ilmastikutingimustes üks enim kasvatatavaid söödakultuure parasvöötme piirkondades, eriti Euroopas. Kliimamuutus süvendab temperatuuri äärmuslikke kõikumisi ja muudab sademete jaotust, mille tagajärjel peavad jahedate ilmastikutingimustega kohanenud heintaimed üha sagedamini ja intensiivsemalt taluma madalaid temperatuure ja põuaperioode. Selline ebasoodsate kliimatingimuste mõju vähendab kasvu ning seega ka biomassi kogunemist ja söödasaaki.

Käesoleva väitekirja eesmärk oli transkriptoomika uuringute abil kindlaks teha põua- ja külmastressi taluvuse mehhanismid ja geenid, et leida kandidaatgeene, millede muutmine oleks kasulik tulevastes aretusprogrammides. Lisaks arendati välja protoplastidel põhinev platvorm, et testida täppismuutmise efektiivsust in vivo enne nende kasutamist raiheina kalluskultuuride stabiilses transformatsioonis.

Erineva põuataluvusega taimi analüüsiti transkriptoomi tasandil, mis näitas, et tundliku genotüübiga taim oli kergest põuast rohkem mõjutatud ja reageeris sellele varem kui taluva genotüübiga raihein. Taluva genotüübiga taimel ei täheldatud lehekasvu vähenemist, nagu tundliku genotüübiga taime puhul. Lisaks sellele arenesid taluva genotüübiga taimel uuritud stressitingimuste hilisemates etappides paremini juured ja toimus edukamalt kutiikula vahade süntees, millele viitas *TSO1* ja *WSD1* ülesreguleerimine. Täiendavalt ilmnesid teatud geenidel nagu *PAT1* spetsiifilised ekspressioonimustrid, mis osutasid nende rollile tundliku genotüübiga taime halvas toimimises kerge põua tingimustes.

Transkriptoomi analüüsi abil hinnati ka ülalkirjeldatud tingimustele erinevalt reageerivate genotüüpide kohanemist külmaga ja külmumisstressi. Täheldati erinevusi diferentsiaalselt ekspresseeruvate geenide arvus – tundlike genotüüpidega taimedel oli see külmaga kohanemise perioodil väiksem kui taluvate genotüüpidega taimedel, mis võib viidata miinuskraadideks valmistumise piiratud võimekusele. See võib olla põhjuseks, miks tundlikel genotüüpidel esines miinustemperatuuriga kokkupuutel suurem elektrolüütide leke kui taluvatel genotüüpidel. Lisaks registreeriti tundlikes genotüüpides madalatel temperatuuridel kõrge ekspressiooniga tuntud külmareaktsiooni negatiivne regulaator *CRPK1*, mis kinnitab veel kord nende genotüüpidega taimede sobimatut reaktsiooni külmale.

Karjamaa raiheina protoplastide isoleerimine optimeeriti nende kasutamiseks testimisplatvormina, mis võimaldab sõeluda genoomi täppismuutmise komponente in vivo. Polüetüleenglükooli abil binaarsete vektoritega transformeeritud protoplastide genoomse DNA analüüs on sobiv meetod, hindamaks insertsioonide ja deletsioonide esinemist, mis on tingitud vektorites kodeeritud juht-RNA-de juhitud nukleaaside aktiivsusest. Insertsioonide ja deletsioonide jälgimine dekomponeerimisanalüüs (TIDE) võimaldab tuvastada täppismuutmise efektiivsust ja mutatsioonide sagedust Sanger-meetodil sekveneeritud amplikonides. Võrreldes teiste meetoditega nagu järgmise põlvkonna sekveneerimine on pakutud meetod kiirem ja kulutõhusam genoomi täppismuutmise analüüs. Transformatsioonivektorite in vivo testimine võib aidata suurendada transformeeritud taime tekkimise tõenäosust ja lühendada soovitud omadustega, näiteks abiootilise stressi parema taluvusega, organismide tekkimiseks vajalikku aega.

Publication I

Sustek-Sánchez F*, Pashapu AR*, Kemešytė V, Kovi MR, Rognli OA, Sarmiento C, Rostoks N, Jaškūnė K **Growth under mild drought: transcriptomics comparison of perennial ryegrass genotypes with differential tolerance to water deficit**
(manuscript submitted)

*Contributed equally

The rules of the publisher prevent the publication of the manuscript of Publication I before acceptance. Official committee members and opponents will be given a copy of the manuscript to enable them to carry out a judicious review of the dissertation.

Publication II

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Transcriptome profiling reveals insight into the cold response of perennial ryegrass genotypes with contrasting freezing tolerance

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ABSTRACT

Low freezing tolerance threatens the survival and productivity of perennial ryegrass under northern climate. In this study, we aimed to identify transcriptional changes in plants subjected to low and freezing temperatures as well as to elucidate differences between tolerant and sensitive genotypes. Response to freezing stress was evaluated in a panel of 160 perennial ryegrass genotypes by measuring electrolyte leakage after exposure to -12 °C and -14 °C for 24 h. Two tolerant and two sensitive genotypes were selected for the transcriptome analysis. Crown tissue samples were collected at six treatments: before the start of cold acclimation (control point), at the start of acclimation, after one week of acclimation, after three weeks of acclimation, after freezing at -5 °C and freezing at -10 °C. A total of 11,125 differentially expressed genes (DEGs) were identified in the sensitive and 12,937 DEGs in the tolerant genotypes, when comparing the control vs. each of the acclimation and freezing treatments, as well as the end of acclimation vs. freezing treatments. Among the identified DEGs 3323 were unique to the sensitive genotypes, 5135 were unique to the tolerant genotypes and 7802 were shared. Genes upregulated during cold acclimation and freezing stress were linked to the MAPK signalling pathway, circadian rhythm, starch and sucrose metabolism, plant-pathogen interaction, carbon fixation, alpha-linoleic acid metabolism, carotenoid metabolism, glyoxylate and dicarboxylate metabolism pathways. Downregulated genes were linked to ATP-dependent chromatin remodelling, fatty acid elongation and DNA replication. The downregulation of fatty acid elongation and glutathione metabolism DEGs could indicate that the studied genotypes respond to cold stress in a novel or not yet well-characterized manner.

Introduction

In the Nordic-Baltic region and temperate latitudes in general, global warming can have a positive aspect for agricultural production and when coupled with proper adaptation management can encourage adoption of new forage crop species as well as increase the productivity (Kemešytė et al., 2023; Olesen et al., 2011; Wiréhn, 2018). On the other hand, climatic fluctuations and weather anomalies may expose perennial forage crops to new abiotic stress types, such as more frequent warm spells in autumn causing unstable snow cover resulting in deacclimation and reacclimation cycles (Jørgensen et al., 2010). These changes negatively affect cold acclimation of perennial forage crops leading to frost injuries or even crop loss (Dalmannsdottir et al., 2017; Kovi et al., 2016; Uleberg et al., 2014). The unfavourable autumn and winter conditions are the main factors limiting the geographical distribution of species and yield stability. However, during evolution, temperate plants have developed cold adaptation strategies when tolerance is gained during autumn via cold acclimation (also known as hardening) process temperatures (Ding et al., 2013; Thomashow, 1999). Low, non-freezing temperatures ranging from +5 °C to 0 °C for at least a 4-week period, increases the efficiency of plant acclimation leading to higher survival rate and yield (Jaškūnė et al., 2022; Rapacz et al., 2014) by inducing

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changes at physiological level via modification of cell wall and membrane fluidity as well as activation of signalling pathways (Tao et al., 1998). Acclimation process starts with the detection of cool temperatures stress followed by increased membrane rigidity, Ca^{2+} influx, initiation of signalling pathways, and the subsequent responses of ICE-CBF-COR transcriptome reprogramming as well as post-transcriptional regulation and posttranslational modification (reviewed in Juurakko et al., 2021; Sustek-Sánchez et al., 2023). When plants experience low-temperature stress, ICE1 can be released from JAZ proteins that are bound by DELLAs, leading to the activation of CBF3 expression. In turn, CBF3 triggers the expression of genes responsive for decreasing the levels of bioactive gibberellic acid (GA) and facilitates the accumulation of DELLAs. C-repeat binding factors (CBFs), also known as dehydration-responsive element-binding proteins (DREBs), are very important in acclimation process as they are responsible for activating the expression of COR genes via binding to cis-element in the promoter of COR genes (Ritonga and Chen, 2020). Another cold response pathways are abscisic acid (ABA) signalling and mitogen-activated protein kinase (MAPK) cascade pathways functioning as conveyors of stress signals from receptors to effectors initiating adaptive processes (Hossain et al., 2010; Moustafa et al., 2014). Most of the current knowledge about gene regulatory networks involved in cold tolerance comes from studies in Arabidopsis. Although many of these networks are evolutionarily conserved, there are large differences between dicots and monocots and between annual and perennial plant species. Thus, there is a need for more research on crop plants, especially perennial crops exposed to the effects of climate change.

Perennial ryegrass (*Lolium perenne* L.) is the most economically important cool-season pasture and turf grass species. It has a large biogeographical distribution, covering nearly whole Europe (Blanco-Pastor et al., 2019), New Zealand and other temperate areas (Chapman et al., 2023). Perennial ryegrass is valued for excellent forage quality, grazing tolerance, rapid establishment and good seed production. However, these superior properties are exhibited under optimal growth conditions while under drought or low temperatures it performs poorly (Aleliūnas et al., 2015; Helgadóttir et al., 2018; Jaškūnė et al., 2020). Several studies have evaluated the mechanisms involved in cold acclimation at physiological, proteomic and metabolomic levels. Perennial ryegrass accessions of contrasting freezing tolerance were studied for proline, water soluble carbohydrates (WSCs) and lipid content in crown tissue during 21 days of cold acclimation (Hoffman et al., 2010). Freezing tolerant plants accumulated more WSCs, especially sucrose, whereas proline level increased equally in both tolerant and sensitive genotypes. Similar experiments were performed using leaf tissue to evaluate the protein content and different metabolites composition of freezing tolerant and sensitive plants (Bocian et al., 2011, 2015). Freezing tolerant genotypes differed from sensitive ones by higher levels of chloroplast proteins (Bocian et al., 2011) and earlier accumulation of proline and asparagine (Bocian et al., 2015). Increased fructan content was measured in the roots and above ground biomass of a freezing tolerant genotype during cold acclimation (Abeynayake et al., 2015b). Cold acclimation followed by freezing stress induces a complex phytohormonal changes in perennial ryegrass; accumulation of trans-zeatin in the crown and root tissue was recorded during cold acclimation and exposure to freezing led to up-regulation of abscisic and jasmonic acid (Prerostova et al., 2021).

Transcriptome analysis is one of the most effective approaches to identify the genes involved in abiotic stress and to describe the regulatory pathways and mechanisms (Aleliūnas et al., 2020; Dong et al., 2020). However, studies on transcriptional level changes under cold stress in perennial ryegrass is still scarce. Ice recrystallization inhibition (IRI), dehydrin (DHN), and cold-regulated (COR) genes were upregulated, and chlorophyll-binding protein genes were downregulated during cold acclimation in a study that utilized Affymetrix Barley1 GeneChip to study differences between cold acclimated and non-acclimated perennial ryegrass plants (Zhang et al., 2017). Genes related to carbohydrate metabolism, photoperiod regulation and signal transduction were differentially expressed in the leaves of freezing tolerant and sensitive genotypes (Abeynayake et al., 2015a; Paina et al., 2014).

In the present research, we utilized perennial ryegrass genotypes with varying freezing tolerance to study transcriptome profiles, aiming for a deeper understanding of the molecular mechanisms underlying the response to cold (low positive temperature) and freezing (below zero temperature) stress. Similar to previous research, we compared transcriptomic responses before and during cold acclimation (at 7, 14, and 21 days). However, unlike prior studies, we also evaluated transcriptomic responses after exposing plants to freezing temperatures (−5 and −10 °C). This study aims to (i) identify differentially expressed genes during cold acclimation and freezing, and (ii) compare the profiles of these genes between freezing-tolerant and freezing-sensitive genotypes, thereby revealing the molecular pathways that differentiate their stress responses.

Materials and methods

Plant material and electrolyte leakage measurement

A collection of 160 perennial ryegrass genotypes was screened for freezing tolerance. The plant material was established by randomly selecting five genotypes from each of the 32 experimental populations and cultivars utilized in a Nordic/Baltic pre-breeding project (Rognli et al., 2018). Each genotype was vegetatively propagated and planted in cell packs filled with peat substrate, with 4 ramets per cell. They were kept in the greenhouse for 21 days, 16/8 h photoperiod, until they were fully established and then moved into growth chambers (Plant Master, Germany), set at 12/12 h photoperiod and 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), for cold acclimation. Acclimation was performed in two steps, starting at 5 °C for 7 days, and continuing at 2 °C for 14 days. At the end of the cold acclimation period, leaf samples of each genotype from the 4 replicates were taken and placed in 25 ml tubes, filled with 2 ml of distilled water. The tubes were randomly placed in the test tube racks and moved into the climate chamber PE 2412 UY-LX (Angelantoni Industrie, Italy) set at 2 °C. Then the temperature was reduced to −6 °C and held constant until it settled down to −6 °C in all racks. The temperature was monitored by inserting temperature probes into one test tube per rack and recording with a KD7 recorder (Lumel, Poland). After reaching the uniform temperature, it was gradually lowered at a rate of 1.2 °C h^{-1} to a target temperature and held for 24 h. The freezing test was repeated twice, with −14 °C as the target temperature of the first experiment (Exp1), while the second (Exp2) had a target temperature of −12 °C. After the freezing test, tubes with the leaves were moved to the growth chamber set to 2 °C and after 24 h, 10 ml of deionized water was added into each tube. The tubes were shaken overnight at 120 rpm and then initial conductivity (C_{ini}) was measured with YSI 3100 conductivity meter with YSI 3253 Glass Dip Cell (YSI Incorporated, USA). The total conductivity (C_{tot}) was measured after autoclaving the samples at 120 °C for 15 min. Electrolyte leakage was estimated as EL = $(C_{ini})/(C_{tot})$ x 100. Freezing tolerance was defined as percentage of EL at the targeted temperatures of −14 °C and −12 °C.

RNA sampling, extraction and sequencing for transcriptome analysis

For the transcriptome analysis, a set of four perennial ryegrass genotypes, consisting of two tolerant and two sensitive were selected based on EL estimation results. Each selected genotype was vegetatively propagated into 20 replicates, consisting of 2–3 ramets, planted in cell packs filled with peat substrate. The established plants were cold acclimated for 21 days as described above. Before exposure to freezing temperatures, the plants were removed from the soil, the roots were washed and trimmed to 5 cm. Each plant was placed into 50 ml test tubes with 5 ml of water and left for adaptation in the freezing chamber

for 10 h at 1 °C. Afterwards the temperature was lowered to - 5 °C at the rate - 1 °C per hour, kept at - 5 °C for 3 h, then the temperature was lowered to - 10 °C. Crown tissue samples were collected at six times points in 3 replicates (Fig. 1) and immediately frozen in liquid nitrogen. T2, T3, T5 and T6 samplings were made one hour after reaching the target temperature. T1 was made a day prior to the start of cold acclimation, and T4 was made right before moving plants to the freezing chamber. First four samplings (T1-T4) were made at 11 a.m. RNA extraction along with DNA digestion was carried out using QIAGEN's RNeasy Plant Mini Kit and RNase-free DNase, according to the manufacturer guidelines. Library preparation and sequencing (20 million paired reads/sample) were outsourced to Novogene Co Ltd (Cambridge, UK).

Alignment, abundance estimation and functional annotation

To obtain high quality reads, the raw reads were inspected using FastQC (0.11.9) (Ward et al., 2020) followed by trimming and adapter removal using fastp (0.23.2) (Chen et al., 2018) with options –length_required 100, –cut_window_size 4, –cut_mean_quality 15. The cleaned reads were then aligned to the reference genome of perennial ryegrass (Ensembl release 59) using hisat2 (2.2.1). The binary alignment/map (bam) files generated from hisat2 were used to extract a transcript level counts matrix with featureCounts from subread (2.0.6) (Liao et al., 2014) by providing the gene transfer format (gtf) file (ensembl release 59). The resulting counts matrix was used for differential expression analysis. For functional annotation, coding regions of the genes (nucleotide sequences) extracted from the genome using gffread (0.12.7) were blasted against protein sequences of perennial ryegrass downloaded from NCBI using diamond (v2.0.15.153) (Buchfink et al., 2021) with options –ultra-sensitive and –evalue 0.00001.

Differential gene expression analysis

The function filterByExpr from the edgeR package was used to filter out genes with low expression. Principal component analysis based on genes retained after filtering was performed using dudi.pca function from R package ade4 (Dray and Dufour, 2007). The quasi-likelihood approach (Lund et al., 2012) was employed to identify differentially expressed genes. Genes involved in cold acclimation were identified by performing contrasts between T1 vs T2, T1 vs T3 and T1 vs T4 followed by contrasts between T1 vs T5, T1 vs T6, T4 vs T5, T4 vs T6, and T5 vs T6 to identify genes involved in freezing stress responses. The above-mentioned contrasts were performed separately in sensitive and tolerant genotypes (e.g. Sen T1 vs Sen T2, Tol T1 vs Tol T2). Furthermore, contrasts were performed at respective treatments between freezing tolerant and sensitive genotypes to identify differences in gene expression between genotypes (e.g. Sen T2 vs Tol T2, Sen T4 vs Tol T4). Only genes with p.adj <0.05 and log fold change ≥ log2(1.5) were considered as differentially expressed. To identify the genes responsible for differences in cold acclimation and freezing tolerance between sensitive and tolerant genotypes, DEGs from the direct comparisons between genotypes were retained only if they were differentially expressed in at least one of the treatment contrasts (T1 vs T2, T1 vs T3, T1 vs T4, T1 vs T5, T1 vs T6, T4 vs T5, T4 vs T6, and T5 vs T6) in both sensitive and tolerant genotypes. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on sets of upregulated and downregulated genes were performed by enrichGO and enrichKEGG functions from R package clusterProfiler (4.10.1) (Wu et al., 2021).

Results

Freezing tolerance assessment

A substantial variation for freezing tolerance parameters was observed among the tested genotypes especially at the target temperature −12 °C (Exp 1). The mean electrolyte leakage (EL%) at −14 °C (Exp2) was higher than that of EL% at −12 °C ($p < 0.0001$, Student's *t*-test). The variation between repeats was very high (Fig. 2), and the correlation between the results of Exp1 and Exp2 was very weak ($r = 0.20$, $p < 0.05$). Selection of the two most freezing tolerant (low EL%) and sensitive (high EL%) genotypes for the subsequent transcriptome analysis was carried out by identifying those genotypes, which were consistently placed in the first quartile and in the fourth quartile in both Exp1 (Figs. 2A, 3A) and Exp2 (Figs. 2B, 3A). The results obtained from the EL experiments corresponded with the clustering observed when performing the principal component analysis based on the DEGs of the sampled plants.

Alignment, functional annotation, and explanatory data analysis

The alignment rate was > 72 % for all samples in the study with a mean alignment rate of ~ 78 %. Homology-based search of coding regions of the genes against the protein sequences of perennial ryegrass retrieved from NCBI's database annotated 35,926 (92 %) genes. After filtering genes with low counts, a total number of 28,535 genes were retained for differential expression analysis and the mean library size was around ~ 17.5 million reads per sample. Principal component analysis (PCA) based on all the genes retained after filtering ordered treatments T1 to T6 from left to right along PC1 (~31 % variation) and

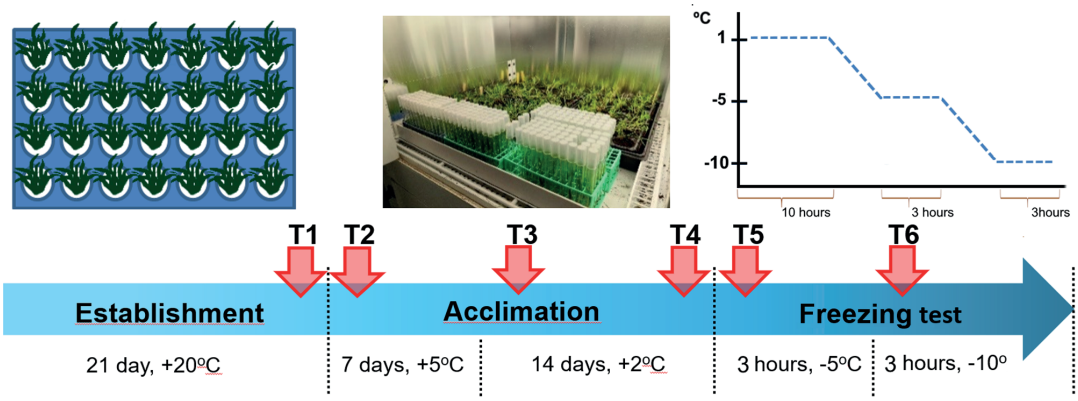

Fig. 1. The design of the cold acclimation and freezing tolerance experiment with indicated time points (red arrows) of RNA sampling, where T1 is control, T2 – beginning of acclimation at +5 °C, T3 – beginning of acclimation at +2 °C, T4 – end of acclimation, T5 – freezing at −5 °C and T6 – freezing at −10 °C.

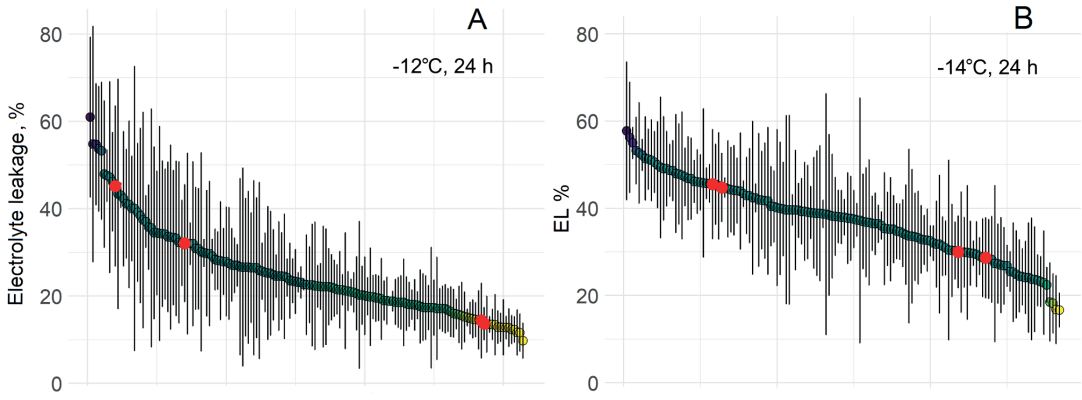

Fig. 2. Electrolyte leakage (EL) variation among perennial ryegrass genotypes ($n = 160$) assessed for freezing tolerance at −12 °C (A) and −14 °C (B). Error bars represent standard deviation (SD). The genotypes selected for the transcriptome analysis are indicated in red.

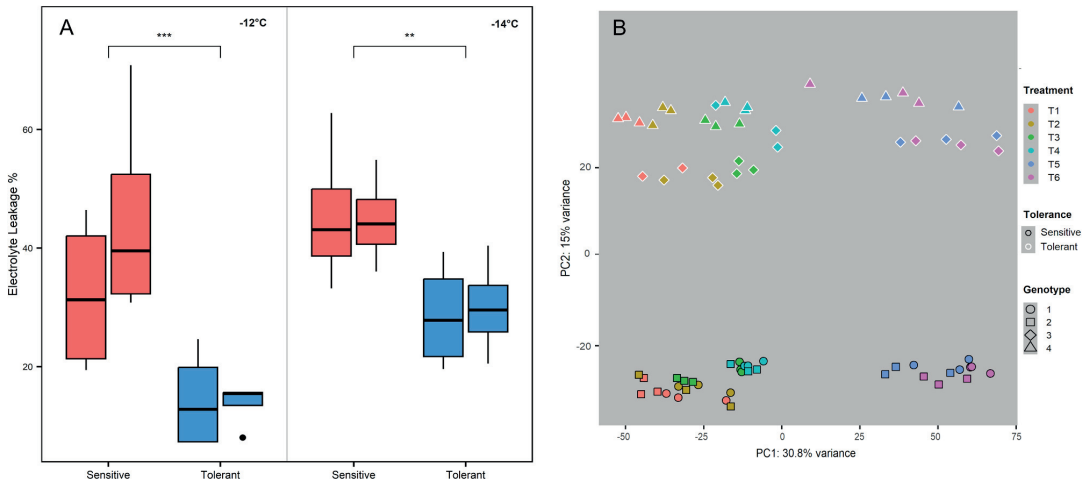

Fig. 3. Electrolyte leakage (EL) and principal component analysis (PCA) of differentially expressed genes of perennial ryegrass genotypes with contrasting freezing tolerance. (A) Variation of EL among freeze sensitive (red) and tolerant (blue) assessed after 24 h at −12 °C and −14 °C. (B) shows the clustering of freeze tolerant and sensitive genotypes along principal component axes 1 and 2.

PC2 explaining 15 % of variation separated the tolerant and sensitive genotypes (Fig. 3B). The first two principal components indicate that experimental factors are the primary source of variation, thus validating the experimental setup.

DEGs during cold acclimation and freezing stress

A total of 11,125 differentially expressed genes (DEGs) were identified in the sensitive and 12,937 DEGs in the tolerant genotype (Fig. 4), when comparing a control (T1) versus each acclimation (T2, T3, T4) and freezing (T5, T6) treatments, as well as the end of acclimation (T4) versus freezing treatments (T5 and T6). Among the identified DEGs 3323 were unique to the sensitive genotype (sensitive specific cold-responsive genes), 5135 were unique to the tolerant genotype (tolerant specific cold-responsive genes) and 7802 were shared between both genotypes (core cold-responsive genes). There were no differentially expressed genes identified at contrast T5 vs T6 neither in the tolerant nor the sensitive genotype. The tolerant genotype had more upregulated and downregulated DEGs compared to the sensitive genotype during cold acclimation (T1 vs T2, T1 vs T3, T1 vs T4) and at the onset of freezing temperatures (T1 vs T5) (Fig. 4B). The number of DEGs was approximately identical at contrasts T4 vs T5, compared to T4 vs T6 in the tolerant genotype, while it was higher at contrast T4 vs T6 compared to T4 vs T5 in the sensitive genotype (Fig. 4B). Several genes encoding proteins and transcription factors (TFs) were differentially expressed in both genotypes and are known to be involved in cold acclimation and freezing tolerance, such as DREB/CBF, cold-shock proteins, late embryogenesis abundant proteins (LEA), heat shock proteins (HSP) and others described in Table 1. KEGG pathway analysis of DEGs revealed that genes upregulated during cold acclimation and freezing stress are linked to the MAPK signalling pathway, circadian rhythm, starch and sucrose metabolism, plant-pathogen interaction, carbon fixation in photosynthetic organisms, alpha-linoleic acid metabolism, carotenoid metabolism, glyoxylate and dicarboxylate metabolism pathways while downregulated genes were linked to ATP-dependent chromatin remodelling, fatty acid elongation and DNA replication (Figs. 5 and 6).

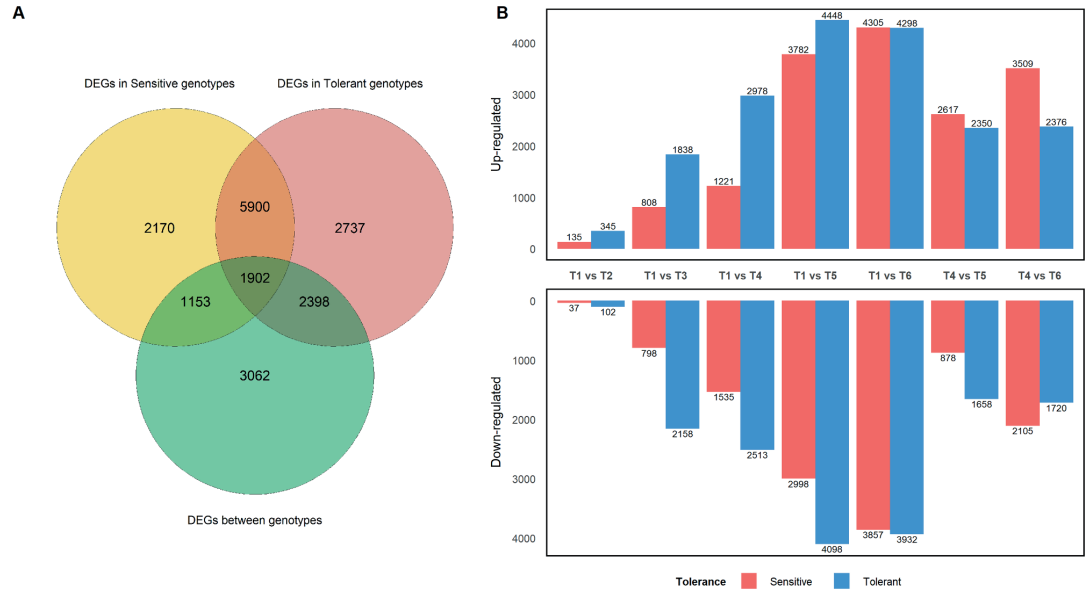

Fig. 4. Differentially expressed genes (DEGs) in the freeze tolerant and sensitive genotypes, where contrasts between genotypes are shown in Figure A, while B) shows number of upregulated and downregulated genes between treatment contrasts in genotypes.

DEGs responsible for differences in freezing tolerance between tolerant and sensitive genotypes

Comparative transcriptomic analysis between sensitive (baseline) and tolerant genotypes identified 4720 (2408 up, 2312 down), 3708 (1902 up, 1806 down), 3921 (1745 up, 2176 down), 4187 (2793 up, 1394 down), 3727 (1900 up, 1827 down) and 4057 (2149 up, 1908 down) differentially expressed genes at T1, T2, T3, T4, T5, and T6 respectively (Supplementary file 1). To identify the genes responsible for the differences in freezing tolerant and sensitive genotypes, DEGs from between genotype contrasts were filtered out based on the core cold-responsive genes (7802 genes) (Fig. 4). After filtering, 926, 644, 752, 640, 682, and 810 genes were retained at contrast T1, T2, T3, T4, T5, and T6 respectively (Supplementary file 2). Further inspection of the filtered gene lists revealed several genes, known for their roles in cold and abiotic stress responses in plants, were highly differentially expressed between the genotypes during cold stress (Fig. 6, Table 1, Supplementary Table 1, Supplementary Fig. 1). Genes coding for cold shock protein CS120-like, cold-regulated 413 protein, dehydrin DHN3-like, HSP, HSF A-2a-like, glycine-rich cell wall structural protein 1, CBL-interacting protein kinase 21-like, LEA 1 subgroup, bHLH 6-like TF, SCREAM 2 TF, MADS-box TF 14-like, MADS-box TF 50-like, common-plant regulatory factor 1-like, b-ZIP TF 23-like, hydrophobic protein OSR8-like and cysteine-rich receptor-like protein kinase 10 are among the few DEGs with elevated expression in tolerant genotypes. In sensitive genotypes, genes encoding DREB 1B-like, NDR1/HIN1-like protein, 36.4 kDa proline-rich protein-like, sucrose 1-fructosyltransferase-like, MYB77-like TF, wall-associated receptor kinase 1-like, calcium-dependent protein kinase (CDPK) 13-like, CDPK 26-like, calcium binding protein KIC-like were observed to have higher expression. Interestingly, a gene coding for cold-responsive protein kinase (CRPK) 1-like is downregulated with the onset of cold in tolerant genotypes but its expression remained stable (not significantly DE) in sensitive genotypes. GO analysis of filtered DEGs sets identified GO terms lipid oxidation (GO:0,034,440) and dioxygenase activity (GO:0,051,213) were enriched among genes with higher expression in sensitive genotypes at T5 and T6 (Supplementary Fig. 2), while GO terms; response to water (GO:0,009,415) and response to acid chemical (GO:0,001,101) were enriched in genes with higher expression in tolerant genotypes at T4.

Discussion

Perennial ryegrass in Europe spans from the Iberian Peninsula to Scandinavia (Blanco-Pastor et al., 2019) and thus is subjected to an array of climatic stressors and their combinations. Though warmer winters have led to less winter damage of forage crops in the Baltic region (Kemešytė et al., 2020, 2023), low freezing tolerance is still an important limiting factor for perennial ryegrass cultivation. Better understanding of the molecular mechanism behind perennial ryegrass cold acclimation and freezing stress response remains an important research field from both a practical and fundamental point of view.

Transcriptomic profile during cold acclimation

To better understand the different pathways that were enriched at certain treatments, the DEGs at basal level (T1) were compared to those of the rest of treatments. The analysis revealed that tolerant genotypes consistently exhibited a higher number of differentially expressed genes (DEGs) during cold acclimation (T1 vs T2, T1 vs T3 and T1 vs T4) than sensitive genotypes (Fig. 4B). In the comparison between T1 and T2 (beginning of cold acclimation), both the sensitive and tolerant genotypes upregulated genes involved in the MAPK signalling pathway. The MAPK pathway plays a crucial role in signal transduction during stress responses and is involved in the calcium signalling response to cold acclimation in different plant species as shown by Jagodzik et al. (2018) and Guo et al. (2018). The genes involved in the arginine and proline metabolism were enriched in the sensitive genotypes, while genes related to the circadian rhythm were enriched in tolerant genotypes. The involvement of the circadian rhythm pathway aligns with findings by Jang et al. (2024), indicating its role in regulating cold-responsive genes like DREB1. Proline is known as an osmoprotectant accumulating during cold acclimation and can be used for energy production under stress conditions (Liang et al., 2013). In L. *perenne*, a previous study observed higher levels of proline in the crown tissue of freezing tolerant plants

Table 1
Genes identified as differentially expressed between the sensitive and tolerant genotypes during cold acclimation and freezing stress.

Gene ID	Description	Role	Higher expression in	DE in contrasts	Ref.
KYUSg_chr2.31927	bHLH 6-like transcription factor (TF)	MYC2 has been shown to be involved in chilling resistance. It positively regulates the expression of CBFs.	Tolerant	T3, T4, T5, T6	10.3389/fpls.2022.868874
KYUSg_chr2.47834	36.4 kDa proline-rich protein-like	EARLI1 can improve membrane or cell wall stability under cold stress.	Tolerant	T3, T4, T5, T6	10.1111/ j.1365–3040.2004.01198.x
KYUSg_chr2.47869	36.4 kDa proline-rich protein-like	EARLI1 can improve membrane or cell wall stability under cold stress.	Sensitive	T1, T2, T3, T4, T5, T6	10.1111/ j.1365–3040.2004.01198.x
KYUSg_chr2.50031	Calcium-dependent protein kinase 13-like	CDPK13 positively regulates cold response in rice.	Sensitive	T3, T4, T5, T6	10.1007/ s00438–007–0220–6
KYUSg_chr3.34	sucrose:sucrose 1-fructosyltransferase-like	1-SST is downregulated in cold tolerant wheat cultivars.	Sensitive	T5, T6	10.1271/bbb.66.2297
KYUSg_chr3.13649	LEA protein 14-A-like, subgroup 2	Proteins belonging to the group II LEA proteins can provide protection against cold in different plant species.	Tolerant	T3, T4	10.1007/ s10725–015–0113–3 10.3390/biom11111662
KYUSg_chr3.42386	SCREAM2-like TF	ICE2 positively regulates cold tolerance.	Tolerant	T1, T2, T3, T4, T5, T6	10.1016/j.gene.2008.10.016
KYUSg_chr4.1396	cold-shock protein CS120-like (dehydrin)	CS120 is involved in the COR signaling pathway.	Tolerant	T2, T3, T4, T5, T6	10.1371/journal. pone.0249975
KYUSg_chr4.1433	cold-shock protein CS120-like (dehydrin)	CS120 is involved in the COR signaling pathway.	Tolerant	T4, T5	10.1371/journal. pone.0249975
KYUSg_chr4.51811	MADS-box TF 50-like	MADS50 is a floral regulator that could be involved in vernalization.	Tolerant	T4, T5, T6	10.1111/ j.1365–313X.2004.02082.x
KYUSg_chr4.8841	cold-regulated 413 protein	COR413 genes positively regulate cold response.	Tolerant	T3, T4	10.1104/pp.102.015255
KYUSg_chr4.9742	MADS-box TF 14-like	MADS14 is involved in the transition between apical and inflorescence meristems.	Tolerant	T1, T2, T3, T4, T5, T6	10.1105/tpc.112.097105
KYUSg_chr5.18506	36.4 kDa proline-rich protein-like	EARLI1 can improve membrane or cell wall stability under cold stress.	Sensitive	T1, T2, T3, T4, T5, T6	10.1111/ j.1365–3040.2004.01198.x
KYUSg_chr5.19513	LEA protein, subgroup 1	Proteins of this subgroup have been shown to be involved in drought stress response.	Tolerant	T3, T4, T5	10.1186/ s12863–017–0596–1
KYUSg_chr5.33969	abscisic acid 8′-hydroxylase 3-like	CYP707A3 regulates degradation of ABA under drought stress response.	Tolerant	T2, T3, T4, T5, T6	10.1111/ j.1365–313X.2006.02683.x
KYUSg_chr5.39012	DREB 1B-like	DREB1B positively regulates cold stress response.	Sensitive	T5, T6	10.1007/ s12041–012–0201–3
KYUSg_chr5.39032	DREB 1B-like	DREB1B positively regulates cold stress response.	Sensitive	T5	10.1007/ s12041–012–0201–3
KYUSg_chr5.42448	hydrophobic protein OSR8-like	The over expression of RCI2 has been shown to provide cold stress tolerance.	Tolerant	T1, T2, T3, T4, T5, T6	10.1007/ s00425–015–2386–1
KYUSg_chr6.14911	glycine-rich cell wall structural protein 1	LpGRP1 is related to cold tolerance after cold acclimation.	Tolerant	T2, T3, T4, T5, T6	10.1007/ s00438–005–0095–3
KYUSg_chr6.3990	Dehydrin DHN3-like	High levels of DHN3 have been shown to provide drought stress tolerance.	Tolerant	T4, T6	10.4161/psb.6.10.17088
KYUSg_chr7.20811	cold-responsive protein kinase 1-like	CRPK1 promotes the degradation of CBF proteins by phosphorylating 14–3–3 proteins.	Sensitive	T5, T6	10.1016/j. molcel.2017.02.016
KYUSg_chr7.28634	beta-1,2-xylosyltransferase XYXT1-like	XYXT1 is involved in the response to cold.	Tolerant	T1, T2, T3, T4, T5, T6	10.1093/pcp/pcy003 10.1016/j. plantsci.2015.03.022
KYUSg_chr7.40936	sucrose:sucrose 1-fructosyltransferase-like	1-SST is downregulated in cold tolerant wheat cultivars.	Tolerant	T1, T2, T3, T4, T5, T6	10.1271/bbb.66.2297
KYUSg_scaffold_6468.345	Calcium-dependent protein kinase 26-like	CPK26 has an unknown role.	Sensitive	T1, T2, T3, T5, T6	10.1016/j. tplants.2012.08.008

after 14 days of cold acclimation but no statistically significant differences after the cold acclimation had finished (after 21 days) (Hoffman et al., 2010). Similarly, other researchers showed that the leaf tissue of freezing tolerant L. *perenne* plants exhibited a higher concentration of proline throughout the cold acclimation process than non-freezing tolerant plants (Bocian et al., 2015). It is therefore surprising that proline metabolism genes were enriched in the sensitive genotypes but not in the tolerant ones. During the last stages of cold acclimation (T3 and T4 treatments) the genes upregulated in tolerant genotypes have been demonstrated to belong to circadian rhythm, starch and sucrose metabolism, ribosome biogenesis, and inositol phosphate metabolism pathways. Similar responses to cold have been reported in *Arabidopsis thaliana* and *Brassica napus* (Cheong et al., 2021; Yan et al., 2022). In these same stages of acclimation (T3 and T4), both the sensitive and tolerant genotypes downregulated genes involved in fatty acid elongation. It has been observed that under cold stress, the roots of maize plants display opposite responses, since they upregulate genes involved in fatty acids synthesis and elongation (Guo et al., 2018). Moreover, in tolerant genotypes, genes involved in glutathione metabolism were enriched among DEGs at T1 vs T3 and T1 vs T4. The role of glutathione in cold acclimation relates to its antioxidant ability to control the excess of ROS molecules resultant from the stress response, and to its capability to post-transcriptionally modify proteins involved in cold acclimation (Dorion et al., 2021). The downregulation of fatty acid elongation and glutathione metabolism DEGs could indicate different responses of perennial ryegrass plants towards cold acclimation than the one reported for other monocots like maize.

Transcriptomic profile during freezing

The comparison between the basal (T1) and freezing stress (T5 and T6) conditions uncover similar responses of the genotypes to freezing temperatures. Both genotypes, sensitive and tolerant, have upregulated genes involved in carbon, sucrose and starch metabolism. Sensitive genotypes have upregulated galactose metabolism genes both in T5 and T6, and during the last stages of cold acclimation (T3 and T4), while in tolerant genotypes the upregulation of this pathway is only present at the lowest freezing temperature (T6). In the sensitive genotypes, the

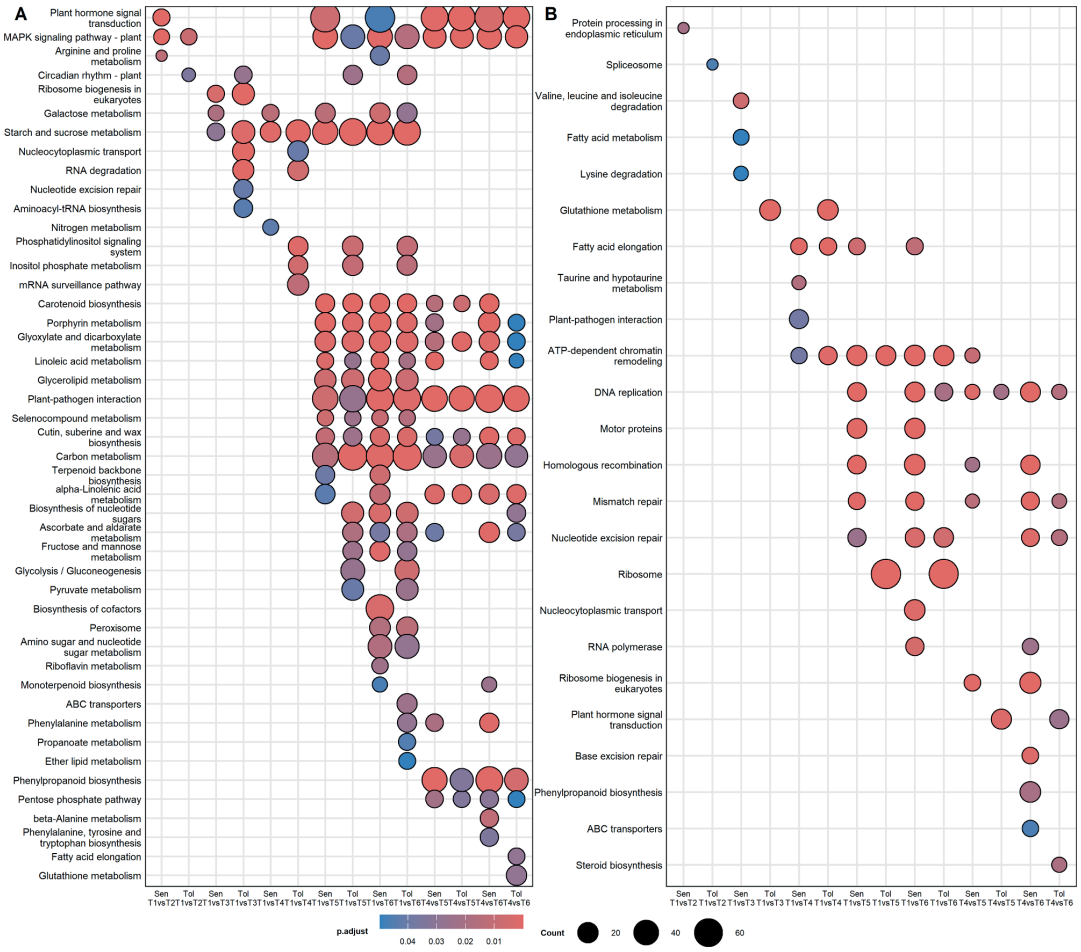

Fig. 5. Enriched KEGG pathways among upregulated genes (A) and downregulated genes (B) during cold acclimation and freezing stress in genotypes with contrasting freezing tolerance, where Sen is the sensitive and Tol is the tolerant perennial ryegrass genotypes. T1 to T6 indicate the treatment, where T1 is control, T2 – beginning of acclimation at +5 °C, T3 – beginning of acclimation at +2 °C, T4 – end of acclimation, T5 – freezing at −5 °C and T6 – freezing at −10 °C.

downregulation of genes involved in cell division and replication, namely the motor protein, ATP-dependent chromatin remodelling, DNA replication and homologous recombination pathways (Kostyrko et al., 2015; Titus and Wadsworth, 2012) is more noticeable in the sensitive genotypes than in tolerant plants. The ATP-dependent chromatin remodelling (in T5 and T6), the DNA replication pathway (in T6) and the ribosome (T5 and T6) pathways are downregulated in the tolerant plants suggesting a decreased cell division and protein synthesis activity in the tolerant genotypes in response to freezing. Additionally, the tolerant genotypes present upregulation of DEGs related to the circadian rhythm in response to freezing stress, similarly to how they behave at the early and mid-stage of cold acclimation. These findings show that tolerant genotypes cease their growth under low-temperature stress during acclimation, which may not be the case with sensitive genotypes. And the opposite, sensitive genotypes have an inadequate cold acclimation process that could undermine their ability to cope with freezing temperatures. In addition, the fact that the tolerant genotypes downregulated DEGs related to pathways previously associated to cold acclimation and freezing tolerance in other plant species, such as the fatty acid elongation and glutathione metabolism pathways, suggests that the studied genotypes of *L. perenne* cope with cold stress in a different, novel or not well-characterized manner.

Functional role of genes identified as differentially expressed between tolerant and sensitive genotypes

Comparative transcriptomic analysis has identified several genes which might be responsible for the differences between the sensitive and tolerant genotypes under low temperature and freezing stress (Fig. 6 and Table 1). The genes coding for ICE2, cold shock protein CS120-like, dehydrin DHN3-like, heat shock protein (HSP) HSF A-2a-like, glutathione S-transferase 1-like, LEA proteins and glycine-rich cell wall structural protein 1 were observed to have significantly higher expression in the tolerant genotypes. These genes are known to play key roles, in the ICE-CBF-COR signalling pathway, protecting cellular structures during stress, enhancing antioxidant capacity, and stabilizing proteins and membranes during freezing stress (Fursova et al., 2009; Hundertmark and Hincha, 2008; Karlson and Imai, 2003; Sustek-Sánchez et al., 2023). In the current study, the tolerant genotypes had significantly lower electrolyte leakages (Fig. 3A) compared to the sensitive

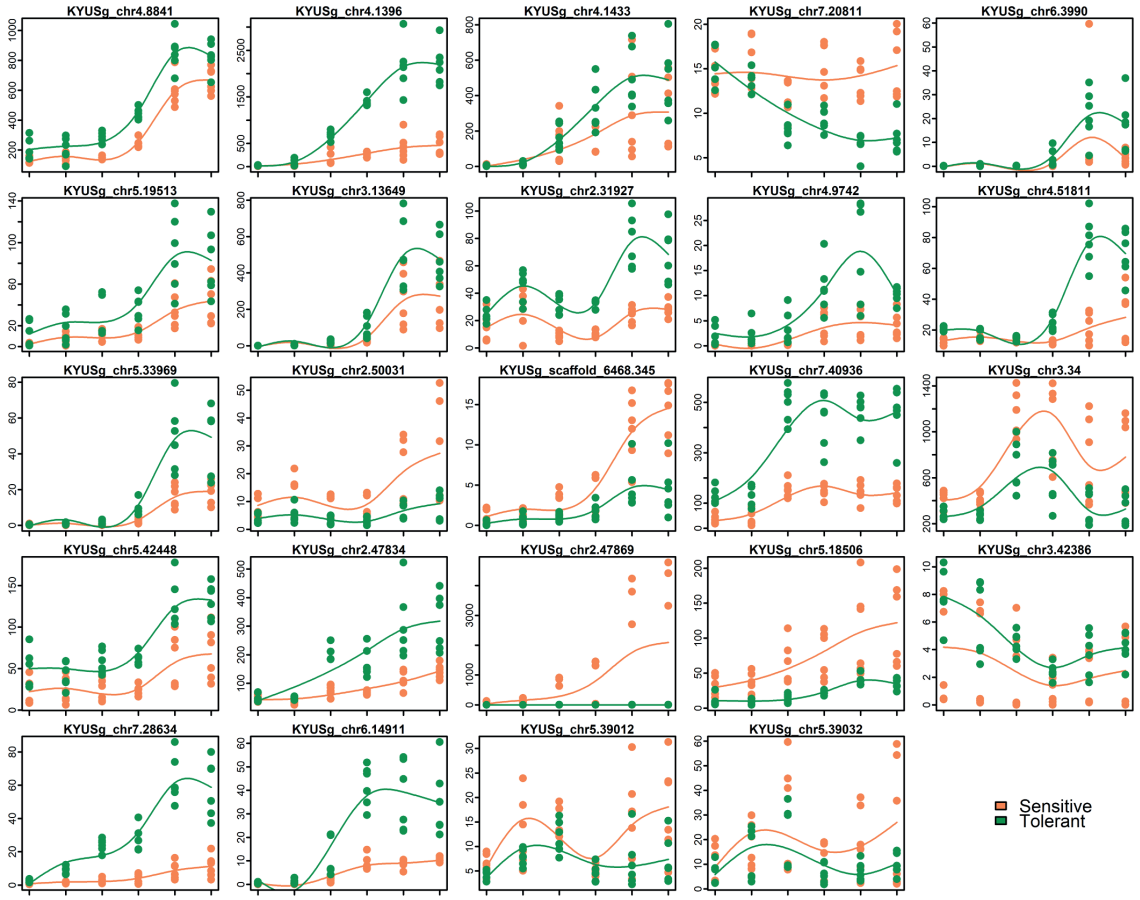

Fig. 6. Some of the genes identified as differentially expressed between the sensitive and tolerant genotypes during cold acclimation and freezing stress. Ticks on the x axis denote treatments (T1 to T6 from left to right) and y axis denotes trimmed mean of M values (TMM) normalized counts per million (CPM).

genotypes, reflecting better cellular integrity under stress conditions. This phenotypic resilience correlates with the robust expression of the aforementioned genes in tolerant genotypes. In contrast, genes coding for DREB 1B-like, beta-fructofuranosidase, and calcium-dependent protein kinase (CDPK) were expressed at a higher level in sensitive genotypes highlighting the contrasting strategies employed by tolerant and sensitive genotypes in response to cold stress. Previous studies have identified DREB1 as a regulator of cold stress responses in Arabidopsis and rice (Liu et al., 1998). When comparing transgenic lines of Arabidopsis with impaired expression of DREB1B with wild-type plants, researchers observed that the survivability of the plants was affected by cold acclimation but that their constitutive freezing tolerance was independent of the DREB1B expression (Novillo et al., 2007). This could be the reason why in our study we observe a higher expression of DREB1B in sensitive genotypes at the beginning of cold acclimation (T2, Fig. 6) than in the tolerant ones, even though they are less resilient towards freezing conditions in terms of their electrolyte leakage. Moreover, genes associated with lipid oxidation, such as acyl-coenzyme A oxidase 4, lipoxygenase 2.3, chloroplastic-like and linoleate 9S-lipoxygenase 3 which upregulate with the onset of freezing temperatures in both genotypes appear to have significant higher expression in sensitive genotypes at T5 and T6 compared to tolerant genotypes (Supp Fig. 3). Over expression of lipoxygenases is linked with increased lipid peroxidation (Lim et al., 2015). Lipid oxidation, while serving as a signalling mechanism can also damage membranes (Alché, 2019; Niki et al., 1991) suggesting us that higher electrolyte leakage in sensitive genotypes could be due to membrane damage as a result of lipid peroxidation. At the beginning of cold acclimation (T2) and at –5 °C (T5), an elevated level of expression was observed of transcription factor MYC2, which is a positive regulator of the ICE-CBF-COR signaling pathway and thus related to freezing tolerance (Song et al., 2022). Higher expression of EARLI1, a lipid transfer protein improving membrane stability under low temperature conditions (Bubier and Schläppi, 2004), was observed in the tolerant genotypes during cold acclimation (T3 and T4) and freezing (T5 and T6), while the sensitive ones expressed it at all stages. Presumably, it may be related to the flowering pattern of sensitive genotypes, as EARLI1 has been reported to respond toward vernalization (Wilkosz and Schläppi, 2000). The gene CDPK13 had elevated expression in both tolerant and sensitive genotypes under freezing temperatures (T5 and T6). Similar responses were shown in a cold tolerant phenotype of rice (Abbasi et al., 2004) and transgenic rice plants overexpressing CDPK13 with improved cold tolerance (Komatsu et al., 2007). However, in our study the sensitive genotypes also had a high expression of CDPK13, suggesting that it may not be related to the different electrolyte leakage observed between the tolerant and sensitive genotypes. 1-SST fructosyltransferase is involved in fructan metabolism.

Its role in cold tolerance was described in perennial ryegrass by Abeynayake et al. (2015a, b) and it was also observed in our studied genotypes. One transcript identified to be translated into 1-SST was differentially expressed in the sensitive plants (KYUSg_chr3.34), while a different transcript had an elevated expression in the tolerant genotypes (KYUSg_chr7.40936). In freezing tolerant wheat plants, the expression levels of this gene have been reported to be downregulated under freezing conditions, suggesting the shift from the synthesis of fructans to simpler sugars (Kawakami and Yoshida, 2002). In both tolerant and sensitive genotypes, the transcript identified as 1-SST was highly expressed at the late stages of cold acclimation (T3 and T4), but the expression decreased with the onset of freezing temperature (T5). Interestingly, expression was higher in the sensitive genotypes than in the tolerant, suggesting they behave similarly to what was reported in winter wheat. The COR413 genes encode both plasma and thylakoid membrane-related proteins involved in cold stress response as part of the ICE-CBF-COR signalling pathway (Breton et al., 2003). Expression of COR413 has been shown to increase in response to cold in *Arabidopsis* which in turn translates into an increased expression of other COR and CBF genes such as COR15 and CBF2 (Hu et al., 2021; Hwarari et al., 2022). This gene had a high expression in the tolerant genotypes during T3 and T4 suggesting its involvement in the cold acclimation of these L. *perenne* genotypes. In the tolerant genotypes, the GRP1 gene had an elevated expression level in all treatments (T2, T3, T4, T5 and T6). This gene has been suggested to be involved in the acquisition of freezing tolerance in L. *perenne* after cold acclimation (Shinozuka et al., 2006) and our results corroborate these findings. Similar results have also been reported in transgenic Arabidopsis plants encoding different GRP genes (including a rice homolog of GRP1) after cold acclimation treatments (Kim et al., 2010). Finally, the CRPK1 gene was also identified to have differential expression between genotypes. CRPK1 has been suggested to be a negative regulator of freezing tolerance and is involved in the degradation of CBF proteins by phosphorylating 14–3–3 proteins that interact with them in the nucleus (Liu et al., 2017). In this study, the expression levels of CRPK1 remained stable throughout the different treatments in the sensitive genotypes, while its expression levels progressively diminished in the tolerant genotypes after the cold acclimation began and continued to decrease in the following treatments. This expression pattern suggests that the sensitive genotypes might have an impaired cold acclimation process, which does not allow the plants to inhibit the degradation of the CBF proteins to achieve freezing tolerance.

Conclusions

The aim of the transcriptomic analysis was to connect the phenotypic response (electrolyte leakage) of freezing tolerant and sensitive perennial ryegrass genotypes with gene expression. The results obtained provide insight into the contrasting response during cold acclimation and freezing stress of the studied genotypes. The phenotypic differences in electrolyte leakage between the tolerant and sensitive genotypes could be attributed to the differences in expression of cold shock protein CS120-like, dehydrin DHN3-like, heat shock protein A-2a-like, LEA proteins, glycine-rich cell wall structural protein 1 and genes associated with lipid oxidation. The sensitive genotypes had in general fewer DEGs compared to tolerant genotypes during cold acclimation and the beginning of freezing stress. The findings imply that sensitive genotypes respond to cold and freezing stress slower resulting into impaired cold acclimation which finally turns into their inability to cope with freezing temperatures. Further investigations should be conducted with the DEGs identified in the study, to better elucidate the pathways responsible for providing freezing tolerance in perennial ryegrass.

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CRediT authorship contribution statement

Akhil Reddy Pashapu: Writing – original draft, Visualization, Formal analysis. **Gražina Statkevičiūtė:** Writing – original draft, Visualization, Investigation, Formal analysis. **Ferenz Sustek-Sánchez:** Writing – review & editing, Writing – original draft. **Mallikarjuna Rao Kovi:** Writing – review & editing, Conceptualization. **Odd Arne Rognli:** Writing – review & editing, Supervision, Conceptualization. **Cecilia Sarmiento:** Writing – review & editing, Writing – original draft. **Nils Rostoks:** Writing – review & editing, Funding acquisition. **Kristina Jaškūnė:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The RNA-seq data generated in this study is available at EMBL-EBI under the accession number E-MTAB-14223.

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Supplementary materials

Appendix. Supplementary materials: The Supplementary Material includes File 1, File 2 and File 3 (containing Supplementary Table 1, Supplementary Fig. 1, Supplementary Fig. 2 and Supplementary Fig. 3).

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100598.

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Improving abiotic stress tolerance of forage grasses – prospects of using genome editing

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Due to an increase in the consumption of food, feed, and fuel and to meet global food security needs for the rapidly growing human population, there is a necessity to obtain high-yielding crops that can adapt to future climate changes. Currently, the main feed source used for ruminant livestock production is forage grasses. In temperate climate zones, perennial grasses grown for feed are widely distributed and tend to suffer under unfavorable environmental conditions. Genome editing has been shown to be an effective tool for the development of abiotic stress-resistant plants. The highly versatile CRISPR-Cas system enables increasingly complex modifications in genomes while maintaining precision and low off-target frequency mutations. In this review, we provide an overview of forage grass species that have been subjected to genome editing. We offer a perspective view on the generation of plants resilient to abiotic stresses. Due to the broad factors contributing to these stresses the review focuses on drought, salt, heat, and cold stresses. The application of new genomic techniques (e.g., CRISPR-Cas) allows addressing several challenges caused by climate change and abiotic stresses for developing forage grass cultivars with improved adaptation to the future climatic conditions. Genome editing will contribute towards developing safe and sustainable food systems.

KEYWORDS

CRISPR, genome editing, gene editing, forage grass, abiotic stress, plant, plant breeding

1 Introduction

Grasses belong to the family of Poaceae, which constitutes the most economically important plant family (Lee et al., 2020; Huang et al., 2022). Grasslands and meadows extend over vast portions of the planet, on land, and even under the sea (Lopez et al., 2022; McSteen and Kellogg, 2022). Their importance in Earth's ecosystems goes beyond their use in fields and pastures. Grassy biomes comprise more than one-quarter of the planet's land area.

Grasses not only provide food, shelter, and building materials for animals and humans, but they also generate oxygen and store carbon (Strömberg and Staver, 2022). This storage, mainly subterranean, contributes towards the fertilization of soils and makes grasslands valuable sinks of CO_2 (Bengtsson et al., 2019; Terrer et al., 2021). Furthermore, grasses are considered more resilient to dryer and warmer conditions than trees. These facts suggest that in the climatic conditions predicted for the future, grasslands could be a better and more robust carbon sink than forests (Dass et al., 2018).

Grass crops provide the most essential dietary food sources globally. From these, forage grasses are the main component used to feed ruminant livestock (FAO, 2018; FAO, 2019). Grasses can be cultivated in less fertile lands compared to other crops. In these zones, normally associated with developing countries (Feller et al., 2012; Kwadzo and Quayson, 2021), animal husbandry and its derivates e.g., dairy production, remain essential (Capstaff and Miller, 2018; Moorby and Fraser, 2021). To cope with the predicted population growth and the consequential increase in food needs, high-yielding crops must be further developed (Raza et al., 2019). To reach food security, the strategies used must avoid causing negative environmental impacts. Synthetic nitrogen-based fertilizers have been important for reaching high yields, nevertheless, their production and usage are a source of massive generation and emission of greenhouse gases (GHGs) (Chai et al., 2019). It is well known that the high concentration of atmospheric GHGs is closely related to climate change. Therefore, the challenge is to increase farming efficiency while reducing the impact of agricultural activity on climate change (Rivero et al., 2021). Importantly, climate change not only directly affects crop productivity but also has indirect and socio-economic impacts, for instance soil fertility, need for irrigation, food demand, policy, rising costs (reviewed in (Raza et al., 2019)).

Grasses usage as forage and as reliable sinks of carbon emissions, call for an improvement in their biomass yield, and their resistance towards the new abiotic and biotic stresses caused by climate change (Giridhar and Samireddypalle, 2015). Especially, plants will have to cope with variations in temperature, water availability, and soil composition (Cushman et al., 2022). Said variations will generate stresses due to heat, cold, drought, and salinity conditions. A promising approach to provide grasses with stress resistance is using genome editing techniques (Bailey-Serres et al., 2019; Dhakate et al., 2022). The first attempts have been performed to use genome editing in forage grasses (Liu et al., 2020b; Weiss et al., 2020; Zhang et al., 2020; Cheng et al., 2021; Zhang et al., 2021; Zhu et al., 2021; Kumar et al., 2022; Wang et al., 2022). This is not an easy task due to their reproductive and genetic characteristics which are difficult to work with. The inability of forage grasses to self-pollinate hinders inbreeding. Additionally, forage grasses have high variability between the genetic background of different individuals. This provides them with a considerable gene pool, responsible for their adaptability and resilience towards environmental changes. Conversely, it creates difficulties for studies focused on identifying the genetic cause of traits or phenotypes of interest (Cropano et al., 2021; Muguerza et al., 2022). There are diverse ways of classifying grasses beyond their taxonomy. For instance, forage grasses can be divided into different types depending on their life cycle and ecotype. In the first case, according to the survival of the plant after going through its reproductive phase, grasses can be considered annual, biannual, or perennial. In terms of their ecotype, grasses can be separated into warm- or cool-season plants if their optimal growth happens during winter or summer, respectively. Importantly, warm-season grasses are C_4 plants, while cool-season grasses are C_3 plants (Moser and Hoveland, 1996; Moser et al., 2004).

In this review, we provide an overview of the main metabolic and molecular changes that plants suffer to cope with the effects of abiotic stress derived from climate change. Additionally, we summarize the actual state of genome editing applications in forage grasses. We propose how genome editing could be used to generate grass plants able to resist these abiotic stresses. Finally, we hypothesize how the new genetic resources and tools can be used to improve forage grass breeding that will help achieve food security in a sustainable way.

2 Cellular and molecular responses to cope with the main abiotic stresses

Extreme temperatures, uncommon precipitation patterns, and deterioration of soils are being observed due to climate change. These environmental consequences have a great impact on agriculture since plants are of sessile nature. The responses used by plants when encountering a stressor aim firstly to achieve acclimation to the new environment and later adaptation to it. Acclimation includes adjusting the physiology and metabolism of a plant to achieve a new state of homeostasis, while adaptation involves both phenotypic and genotypic alterations. Acclimation mediates quick responses to ensure the survival of a plant, whereas adaptation is considered an evolutionary and lengthy process whose goal is to preserve a population. Additionally, plants undergo epigenetic modifications when a stressing event happens (Guarino et al., 2022). Plants must cope with new and more extreme conditions, which lead to different abiotic and biotic stresses than those commonly present in their biomes (Sugimoto and Nowack, 2022). Abiotic stresses are those derived from the physical and chemical factors of an environment and are independent of living organisms (He et al., 2018). As a response to these environmental alterations, plants undergo morphological, metabolic, and physiological changes. In this review, we will focus on drought, salinity, cold, and heat stress responses at the cellular and molecular levels. These are not the only abiotic conditions that will vary due to climate change, but they represent some of the major alterations that will result from it (He et al., 2018; Villalobos-López et al., 2022). The stresses discussed in this review have a significant impact on the growth and development of plants, which is directly connected to crops' yield and profitability (Bita and Gerats, 2013; Bulgari et al., 2019).

Even though the abiotic stresses will be described separately, in nature they tend to interact producing greater effects than individually. Therefore, plants normally must acclimate to a combination of stresses. This should not be ignored when designing strategies to improve crops' tolerance to stress (Pascual et al., 2022).

2.1 Temperature conditions

One of the main effect of climate change is the alteration of temperature conditions (Pörtner et al., 2022). Temperature affects and limits plant growth and development directly (Loka et al., 2019). Therefore, it has a great impact on crop yield which is associated with food security (FAO, 2018; FAO, 2019; Pörtner et al., 2022). It is considered that there are two abiotic stresses derived from temperature variations: heat and cold stress.

2.1.1 Heat stress

As a direct consequence of climate change, global warming has led to steady and yearly temperature increase. Even in temperate zone it has become common to experience warmer seasons with particularly extreme temperatures during summer. Hence, heat waves have increased worldwide causing heat stress for plants (Jagadish et al., 2021). Heat stress appears with sudden increases in temperature, 10 or 15°C above usual conditions (Liu et al., 2020a), and its consequence depends on the plant genotype and ecotype, on the level of incremented temperature, and on the length of the stress (Hasanuzzaman et al., 2013; Wang et al., 2018). Plants may survive heat stress through heat-avoidance or heat-tolerance mechanisms (Aleem et al., 2020). The avoidance processes intend to ensure the survival of a plant, for example altering its leaf orientation or regulating its stomatal conductance, while heat-tolerance mechanisms are related to the plant's ability to maintain its growth under heat stress. These processes involve the synthesis and regulation of different enzymes and other proteins (Hasanuzzaman et al., 2013). Plants primary sensing mechanism towards heat stress is located in the plasma membrane of cells. These membranes become more fluid and permeable under heat stress, which activates heat sensor proteins. It is believed that these heat sensors are, or interact, with calcium channels (Bourgine and Guihur, 2021). Calcium is known to be a key molecule involved in the activation of diverse stress responses mechanisms (Xu et al., 2022). Different transmembrane proteins related to calcium transport have been proposed to act as heat sensors. Members of the Annexin gene family, the protein Synaptotagmin A (SYTA) in *Arabidospis thaliana* (L.) Heynh. and the Cyclic Nucleotide-Gated Channels (CNGCs) are examples of heat sensor proteins from plants (DeFalco et al., 2016; Yan et al., 2017; Wu et al., 2022). The CNGCs are cation channels that regulate the entrance of ions, e.g., Ca^{2+}, into the cytosol from the apoplast and have a calmodulin-binding domain in their cytosolic region. This suggests that increased levels of cytosolic Ca^{2+} trigger an unknown signaling cascade that mediates the accumulation of heat-shock proteins (HSPs) (Bourgine and Guihur, 2021). In rice, the induced loss of function of two of these CNGCs proteins, OsCNGC14 and OsCNGC16, showed that mutant plants exhibited reduced survival when exposed to both heat and cold stresses. This concurs with the observed role of CNGCs in heat stress signaling and shows that temperature stresses have overlapping signaling mechanisms (Cui et al., 2020). The abrupt changes derived from heat stress can degrade cellular components, altering the composition of membranes and denaturing proteins. Moreover, oxidative stress is also a common result of abiotic stresses. In consequence, the production of reactive oxygen species (ROS) increases. ROS can be generated in different cellular compartments, such as peroxisomes, mitochondria, and chloroplasts (Hasanuzzaman et al., 2020). These molecules are very toxic and can end up inducing cell death due to damage to proteins, cell membranes, and even DNA (Singh et al., 2019). To avoid drastic consequences, cells induce the synthesis of HSPs and heat-shock transcription factors (HSFs). In response to heat stress, these transcription factors bind the heat-shock elements (HSEs) that are conserved regions of the HSPs genes. This leads to increased levels of HSPs in the cells, which aims to preserve the integrity of cell proteins by preventing their misfolding and aggregation thanks to the chaperoning role of HSPs (Krishna, 2004). The overexpression of *Lolium arundinaceum* (Schreb.) Darbysh. *heat stress transcription factor A2c* (*HsfA2c*) produced plants tolerant to heat stress (Wang et al., 2017). In addition, to prevent damage from oxidative stress plants can use different antioxidant enzymes like peroxidase and catalase. The plant species and ecotype determine which enzymes will be responsible for coping with oxidative stress (Hasanuzzaman et al., 2020). Importantly, metabolic changes, like alterations in enzymes' activity, also occur due to heat stress. In plants, for example, the oxygenase activity of rubisco rises, leading to more photorespiration and therefore reduced carbon fixation and photosynthesis. Furthermore, heat stress alters the degradation and synthesis of carotenoids and chlorophyll that causes a more pronounced decrease in photosynthetic activity (Loka et al., 2019).

2.1.2 Low temperature tolerance and winter hardiness

Winter survival of forage grasses is a very complex trait determined by the interaction of abiotic stresses like low temperature, frost, desiccation, water logging, ice-encasement and snow cover, which also can cause biotic stress by low-temperature fungi (Rognli, 2013). Winter hardiness, persistency and stable high yields are limiting factors for forage grass production in temperate regions. Short growing seasons with long days, the long winter with short days and low light intensity cause stressful conditions for perennial plants. Cold acclimation, tolerance to freezing and ice-encasement are crucial components of winter survival. Plant species from temperate climates, which are frequently exposed to sub-zero temperatures have developed advanced mechanisms to cope with extended periods of cold during winters. These plant species, when exposed to low but non-lethal temperatures, increase their freezing tolerance through a process called cold acclimation (Thomashow, 1999; Chinnusamy et al., 2007). Most forage grass species and winter-types of cereals need vernalization, i.e., the induction of flowering when exposed to low temperatures (Fjellheim et al., 2014). During autumn the plants produce only leaves until the vernalization requirement is met and the tillers switch from vegetative to generative growth. However, stem elongation and flowering need long days and normal growth temperatures and will not happen until spring (Heide, 1994).

Long duration of ice cover (ice-encasement) is the major cause of winter damage (Gudleifsson, 2009). Warm spells in winter cause snowmelt, which then form non-permeable ice layers when the temperature returns to below zero, causing anoxic conditions for plants (Larsen, 1994). Though freezing tolerance gives a good estimate for winter hardiness, the correlation between freezing

tolerance and tolerance to ice-encasement is relatively less known (Andrews and Gudleifsson, 1983). Studies by Gudleifsson and colleagues showed a weak correlation (r=0.36) between freezing tolerance and ice-encasement (Gudleifsson et al., 1986).

Freezing tolerance is a complex dynamic trait which requires a fine-tuned coordinated response at the physiological and sub-cellular level in relation to environmental cues to induce physiological, biochemical, and metabolic changes (Maruyama et al., 2014; Nakaminami et al., 2014). Many of these resulting cold-associated changes are mainly due to changes in gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006; Thomashow, 2010; Maruyama et al., 2014). Temperature, light, and a complex interaction of these two variables are key factors driving the process of cold acclimation and determining the extent of freezing tolerance acquired (Gray et al., 1997; Janda et al., 2014; Rapacz et al., 2014; Dalmannsdottir et al., 2017).

With the increase in autumn temperatures, cold acclimation will occur during late autumn or early winter under different irradiance levels than normal conditions (Dalmannsdottir et al., 2016; Dalmannsdottir et al., 2017). Water logging conditions as a result of the heavy precipitation in autumn during cold acclimation may also negatively affect cold acclimation and freezing tolerance (Jørgensen et al., 2020). Winter survival under novel climate conditions is likely to be determined by the ability to cold acclimate at low non-freezing temperatures, resist deacclimation during short warm spells in mid-winters and re-acclimation when the temperatures drop again after the warm spells (Kovi et al., 2016; Rapacz et al., 2017; Jaškūnė et al., 2022a).

The *inducer of CBF expression* (*ICE*), *C-repeat binding factor* (*CBF*) and *cold-responsive* (*COR*) genes are considered the master regulators of plants' response to cold (Hwarari et al., 2022). They form the ICE-CBF-COR signaling cascade, which is known to play a key role in freezing tolerance and remains the best-characterized pathway to date (Thomashow, 2010; Ding et al., 2019b). CBF regulon consisting of genes *CBF1*, *CBF2* and *CBF3* amongst others contributes to acclimation to cold temperatures (Park et al., 2018). These genes were first studied in Arabidopsis and encode transcription factors that bind to dehydration responsive genes, as well as those with an early response to cold and dehydration (Galiba et al., 2009). Other important proteins contributing to winter survival are dehydrins (DHNs) or group 2 Late Embryogenesis Abundant (LEA) proteins. Many grass species are tolerant to freezing by upregulating *DHN* genes (Liu et al., 2017). Dehydrins are often regulated by CBF cold-responsive pathways. The C-repeat/dehydration-responsive element binding factors (CBF/DREB) are transcription factors that recognize and bind to the dehydration-responsive element/C-repeat (DRE/CRT) elements in the promoter of *COR* genes (Vazquez-Hernandez et al., 2017). The transcriptome analysis in *Elymus nutans* Griseb. showed that the genes encoding LEA14-A, cold-regulated plasma membrane protein COR413PM, cold-responsive protein COR14a and dehydrin COR410 had higher transcriptional abundance in a genotype with higher tolerance to cold (Fu et al., 2016). Further, quantitative trait loci (QTLs) for winter survival, frost and drought tolerance have been mapped in meadow fescue (*Lolium pratense* (Huds.) Darbysh.). Several of the QTLs were located in the same chromosomal regions as QTLs and genes in Triticeae species, notably DHNs, CBFs and vernalization response genes. The major frost tolerance/winter survival QTL co-located with the position of the *CBF6* gene. Some of the winter survival QTLs co-located with frost tolerance QTLs, others with drought QTLs, while some were unique and most likely this was due to segregation for genes affecting seasonal adaptation, e.g., photoperiodic sensitivity (Alm et al., 2011).

In addition, perennial grass species produce water soluble carbohydrates, such as fructans and raffinose family oligosaccharides during cold acclimation (Bhowmik et al., 2006; Abeynayake et al., 2015). Fructans are an important energy source found in temperate forage grasses. They are synthesized from sucrose and can be defined as storage carbohydrates that are non-structural (Waterhouse and Chatterton, 1993). Fructans are stored in vacuoles and will either have linear or branched fructose polymers with glycosidic bonds to sucrose (Valluru and Van den Ende, 2008). The linear polyfructose molecules tend to accumulate in plants either as an addition to or instead of starch (Chalmers et al., 2005). The levels of fructan in wintering plants are involved in freezing tolerance and they are important for survival during winter and regeneration or sprouting of tissues in spring, being an important sugar supply (Yoshida, 2021). Accumulation of fructans involves fructosyltransferases, invertases and fructan exohydrolases, which are regulated tightly and moreover, their genes have been characterized and isolated (Chalmers et al., 2005; Wu et al., 2021)

2.2 Drought

Drought is one of the main environmental factors limiting crop productivity and predicted climate change shifts in the future will result in temperature increase and change in precipitation patterns (Pörtner et al., 2022). In the semiarid regions, plants have evolved defense mechanisms allowing them to cope with stressful environments and survive prolonged desiccation. These mechanisms include an elaborated antioxidant defense system and complex gene expression programs, ensuring transcription and translation of LEA proteins, heat shock proteins, and other stress-responsive genes, as well as metabolic modulations consisting of various phytohormones and phytochemicals (Farrant et al., 2015; VanBuren et al., 2017; Hilhorst et al., 2018; Oliver et al., 2020). Annual crops escape the limited water conditions by completing their reproductive cycle producing seeds. While annuals can ensure the survival of species *via* seeds, perennial crops must cope with water shortage using drought tolerance and avoidance strategies (Kooyers, 2015; Loka et al., 2019). Plants avoid drought by reducing transpiration and maintaining or even increasing water uptake resulting in postponed tissue dehydration. In contrast, drought tolerant perennial crops experiencing stress survive by suspending shoot growth leading to leaf desiccation. However, the crowns of the plants stay vigorous and recover under adequate rainfall. The latter two strategies are of particular importance in forage crops because they are expected to be high yielding under mild stress and to quickly recover after it. Recent studies on vegetative desiccation tolerance have linked this mechanism to seed-development processes, by showing increased expression of seed-related genes in vegetative tissues during drying (Pardo et al., 2020). The finding suggests that desiccation and water-deficit tolerance mechanisms in grasses derive

from an alternative use or "rewiring" of seed-development pathways. Unraveling the key players involved in this mechanism could be a significant step towards engineering the resurrection trait into drought tolerant forage crops.

Compared to semiarid regions, the typical mild summer drought of temperate zones does not threaten crop survival but causes a significant yield penalty (Moore and Lobell, 2015; Ergon et al., 2018). The strategies result in reduction of aboveground biomass growth and accumulation, which is one of the most agronomically important traits to achieve. Genotypes adapted to water deficit might maintain growth, and under temporary drought scenario they might be considered as competitive in terms of stable biomass accumulation (Jaškūnė et al., 2020). The limited water availability triggers responses at the whole-plant, tissue, cellular and molecular levels (Farooq et al., 2009; He et al., 2018). The perceived stress signal is converted to increased levels of abscisic acid (ABA) production and accumulation in stomatal guard cells which regulate transpiration through stomata closure and thus conserve water in tissues (Wilkinson and Davies, 2010; Lee and Luan, 2012). However, this type of water loss prevention negatively affects the photosynthetic activity and this in turn results in a slowdown of growth and, under prolonged water shortage, growth halt (Farooq et al., 2009). Although ABA negatively impacts the aboveground biomass accumulation, at the same time it has an opposite effect on growth and development of roots that largely help to overcome stress (Saab et al., 1990; Li et al., 2017; Khadka et al., 2019). Nevertheless, improving forage crops for superior yield through ABA-induced drought adaptation remains a great challenge because of ABA mediated stomatal closure leading to reduced carbon gain and ABA-induced senescence (Sah et al., 2016). Another consequence of drought stress in plants is overproduction of ROS causing an oxidative stress which in turn results in cellular membrane damage, imbalance of ions and oxidation of bioactive molecules (Hussain et al., 2016; Hussain et al., 2018).

ABA also plays an important role in inducing the protective role of DHNs. Dehydrins are a subfamily of group 2 LEA proteins that accumulate during late stages of seed development, when plant water content often decreases. In addition, DHNs accumulate in vegetative tissues that are exposed to various stress factors related to dehydration (drought, high salinity, low temperatures, wounding) (Svensson et al., 2002). Hundreds of DHN genes have been sequenced in both dicotyledonous and monocotyledonous plant species (Kosová et al., 2019). The regulation of these genes involves Ca^{2+} signaling pathways as well as ABA and mitogen-activated protein kinase (MAPK) cascades. Dehydrins help to detoxify ROS binding to metal ions and scavenging ROS through oxidative modification. Importantly, the characteristic lysine-rich K-segment of dehydrins displays high membrane affinity. DHNs are known to bind and to protect membranes and even DNA from potential damaging caused by adverse environment. It has been shown that DHNs interact with plasma membrane intrinsic proteins that are important members of the aquaporin family (Liu et al., 2017; Sun et al., 2021). The coordination of intracellular functions, including stress response, depends on the flow of information from the nucleus to cell organelles and back. The expression of many nuclear stress response genes is regulated by 3′-phosphoadenosine 5′-phosphate (PAP), known as a key player in chloroplast stress retrograde signaling, which accumulates during drought, salinity and intensive light stress (Pornsiriwong et al., 2017). The concentrations of PAP are regulated by phosphatase SAL1, which dephosphorylates PAP to Adenosine monophosphate (AMP) and thus reduces PAP levels (Estavillo et al., 2011). The studies on *TaSal1* knockout wheat mutants obtained using CRISPR-Cas9 confirmed PAP accumulation, resulting in enhanced stress signaling and induced stomatal closure. Consequently, mutant plants had bent stem and rolled-leaf phenotype with better regulation of stomatal closure and seed germination (Abdallah et al., 2022).

2.3 Salinity

Salt stress is considered one of the most devastating environmental stresses that limits the productivity and quality of agricultural crops worldwide. Nowadays, over 20% of the world's cultivable lands are affected by salinity stress and due to climate change, resulting in precipitation variation and temperature increase, these areas are continuously expanding (Qadir et al., 2014).

During the process of soil salinization, an excessive increase in water-soluble salts occurs. The most common cations found in saline soils are Na^+, Ca^{2+}, and Mg^{2+}, whereas chloride, sulfates, and carbonates are the main source of anions. The high concentration of dissolved salts in the root zone reduces the osmotic potential difference between the soil and roots, which limits water uptake in plants, causing physiological water deficiency and malabsorption of essential elements (Farooq et al., 2022). The toxic effect of a high concentration of Na^+ is the most prominent one – Na^+ is not needed for plant metabolism, whereas it competes for binding sites with K^+ that is essential for many cellular functions (Tester and Davenport, 2003).

In cells, exposition to salt stress primarily induces osmotic stress and ionic stress. Sensing salt ions and hyperosmolality triggers Ca^{2+} accumulation in the cytosol, activation of ROS signaling, and alteration of membrane phospholipid composition. These signals change phytohormone signaling, cytoskeleton dynamics, and the cell wall structure. Moreover, various physiological and molecular changes inhibit photosynthesis and alter sugar signaling, which may lead to plant growth retention (Zhao et al., 2021).

Several Na^+-binding molecules have been demonstrated to act as sensors able to respond and signal an excess of Na^+ (Shabala et al., 2015). The best-studied of them is the hyperosmolality-gated calcium-permeable channel family OSCA that has been identified in many species, including important cereals (Han et al., 2022b; She et al., 2022).

The environment-triggered Ca^{2+} influx signal in the cytoplasm is received by Ca^{2+}-sensing proteins. Among those, calcineurin B-like proteins (CBLs) are responsible for maintaining the ion transport and homeostasis through interactions with the serine/threonine protein kinases (CIPKs) which activate Na^+, K^+, H^+, NO^{3-}, NH^{4+} and Mg^{2+} transporters located in different cellular membranes. In addition, regulation of ROS and ABA signaling is also modulated by CBL-CIPK complexes (Ma et al., 2020). Regulation of Na^+ transport from cytosol to the apoplast is mediated by the salt overly sensitive (SOS) pathway where the specific complexes of CBLs-CIPKs interact with Na^+/H^+ antiporter SOS1 that removes excessive Na^+. Another CBL-CIPK complex activates Na^+/H^+ exchange transporter 1 located in the

vacuole tonoplast to transport the excess of Na^+ to that organelle (Ma et al., 2020). The CBL and CIPK encoding genes seem to be conserved among dicots and monocots (Martínez-Atienza et al., 2007; Kanwar et al., 2014). Sequestering of the ions into vacuoles helps to avoid stress but needs the osmotic potential adjustment in the cytosol by the accumulation of osmotically active substances such as polyols, amides and amino acids, soluble carbohydrates, and quaternary ammonium compounds. The toxic and osmotic effects of salt ions in the cytoplasm are usually reached by scavenging ROS by antioxidant enzymes that also help to tolerate the toxic effects of salt ions (Flowers and Colmer, 2008).

Other early events in salt stress response include rise of cyclic nucleotides (e.g., cGMP) and ROS. The cGMP inhibits Na^+ influx *via* non-selective ion channel. In addition, rise in cGMP and ROS induces transcriptional regulation that can activate MAPK cascades. Rise in expression of MAPKs leads to increased osmolyte synthesis to alleviate salt-induced osmotic stress. Osmolytes are also a signal for production of ABA, regulating stomatal closure and therefore osmotic homeostasis and water balance (Zhao et al., 2021). Salt stress-induced accumulation of ABA activates the sucrose non-fermenting-1 related protein kinases 2 (SnRK2s). In turn, activated MAPKs and SnRK2s transduce signals to downstream transcription factors to induce the expression of stress-responsive genes (Zhao et al., 2020).

The ability to resist saline environments differs remarkably among plants. Non-halophytic plants (i.e., glycophytes) are sensitive to salinity stress, and their growth and development are hampered by a salinized environment. However, glycophytes exhibit natural variation in their salinity tolerance. Such variation often relies on an allelic variation of genes involved in salinity stress response (Jamil et al., 2011). For example, it has been noticed that under salt treatment to reduce sodium influx in response to osmotic stress, an aquaporin, a cation antiporter, and a calcium-transporting ATPase were downregulated, while a manganese transporter and a vacuolar-type proton ATPase subunit were upregulated in the roots of a salt-tolerant accession of *Poa pratensis* L. when compared to a susceptible accession of *P. pratensis* (Bushman et al., 2016).

Halophytic plants have adapted to salinized environments and they show stimulation of growth enhancement and productivity at moderate salinity (50–250 mM NaCl) (Flowers and Colmer, 2008). These plant species have developed specific mechanisms that regulate internal salt load, e.g., many have developed specialized salt glands which excrete ions on the leaf surface. Such structures are mainly characteristic of C4 grasses belonging to the tribes Chlorideae, Sporoboleae and Aeluropodeae. Other halophytes, including as well C4 grasses (e.g., *Paspalum vaginatum* Sw.), use bladder-like protrusions from epidermal cells into which ions are sequestered and accumulated until these cells senesce and die (Chavarria et al., 2020; Spiekerman and Devos, 2020). The number and density of salt glands or salt bladders depends on salt concentration in the soil during plant growth indicating the dynamic adaptation to environmental conditions (Flowers and Colmer, 2008).

Identification of genetic components and their variance underlying salinity tolerance is a useful source for plant breeders (Zhai et al., 2020). The overexpression of several halophytic genes in glycophytic recipients has been demonstrated to enhance abiotic stress tolerance (Mishra and Tanna, 2017). An increasing number of transcriptomic studies from salt-tolerant non-halophytic and halophytic grasses grown under different salinity conditions will help to elucidate the gene networking process behind the effective salinity response (Xu et al., 2020; Mann et al., 2021; Vaziriyeganeh et al., 2021).

3 Genome editing: A tool for developing stress resistant forage grasses

The biggest challenge for agriculture nowadays is to obtain plants that are resilient to adverse environmental conditions, and at the same time provide enough yield to fulfill food and feed security in a sustainable way. In the case of perennial forage grasses, yield is determined by repeated harvesting of herbage over as many years as possible. Therefore, forage grass genotypes with improved survival and growth under abiotic stress conditions are needed.

Genome editing tools have proven to be useful for achieving such aims, especially the Nobel prize-winning discovery of application of RNA-directed Cas9 nuclease for genome editing (Gasiunas et al., 2012; Jinek et al., 2012) abbreviated as CRISPR-Cas9. Although this editing strategy was immediately applied in model and crop plants, almost ten years ago (Feng et al., 2013; Jiang et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013), not much has been achieved in the forage grasses landscape. The European GMO database EUGENIUS lists only green foxtail (*Setaria viridis* (L.) P. Beauv.) line 193-31 that has been modified using CRISPR-Cas9 mediated mutagenesis. The expressed CRISPR-Cas9 system targeted the coding region of the *S. viridis* homolog of the *Zea mays* L. *Indeterminate 1* (*ID1*) gene, which promotes flowering in maize. The deactivation of the homolog in *S. viridis* led to delayed flowering. In the knockout line 193-31, the CRISPR-Cas9 DNA construct was segregated away (GE Setaria viridis molecular characterization details, n.d).

To find out how many publications have been released showing edited genes in forage grasses, a search was carried out in the following databases: Scopus, Web of Science, Google scholar and PubMed. The search included the scientific or the common names of 47 grass species (Supplementary Table 1) or the name of each of the 12 subfamilies of Poaceae and, in addition, one of the following terms: “CRISPR”, “genome editing”, “genome editing”. The outcome of the search is shown in Table 1. The genome of only six species, three annual grasses and three perennial ones, all growing in temperate regions, has been targeted with CRISPR-Cas tools. Genome editing in *S. viridis*, a model plant for C4 grasses, has been reported three times. Most of the work has been done by knocking out a single gene using the easiest genome editing approach, i.e., CRISPR-Cas9.

CRISPR-Cas9 as a system for carrying out simple mutations (indels: insertions/deletions) that change the reading frame of a coding region and therefore generate knockouts, is straightforward and still mainly used for functional genomics. It consists of two main components: the Cas9 nuclease from *Streptococcus pyogenes* and the short guide RNA (gRNA) that targets the DNA sequence of interest. Designing the gRNA with precision enables the simultaneous mutations of all alleles of a gene in a polyploid plant, as it was the case for *Panicum virgatum* L. (tetraploid) and *Lolium arundinaceum* (allohexaploid, Table 1). Specific genes that have been knocked-out in

TABLE 1 Genome editing in forage grasses.

Species	Common name	Biome	Life cycle	Editing system	Publication
Lolium multiflorum	Italian ryegrass	Temperate	Annual	CRISPR-Cas9	(Zhang et al., 2020)
Lolium perenne	Perennial ryegrass	Temperate	Perennial	CRISPR-Cas9	(Zhang et al., 2020; Kumar et al., 2022)
Panicum virgatum	Switchgrass	Temperate	Perennial	CRISPR-Cas9	(Liu et al., 2020b)
*Lolium arundinaceum**	Tall fescue	Temperate	Perennial	CRISPR-Cas9/Cas12a	(Zhang et al., 2021)
Setaria italica	Foxtail millet	Temperate	Annual	CRISPR-Cas9	(Cheng et al., 2021; Zhang et al., 2021b; Wang et al., 2022)
Setaria viridis	Green foxtail	Temperate	Annual	CRISPR-Cas9_Trex2	(Weiss et al., 2020)
				CRISPR-Cas9	(Zhu et al., 2021; Experimental releases of GM Plants)

*Festuca arundinacea.

forage grasses are related to flowering (*phytochrome C—PHYC—*of *Setaria italica* (L.) P.Beauv. and *floral organ number 2—FON2—*of *S. viridis*), tillering and branching (*teosinte branched 1—tb1a* and *tb1b—* of *Panicum virgatum*), meiosis (*disrupted meitoic cDNA 1—DMC1—* of *Lolium multiflorum* Lam.), haploid induction (*matrilineal—MTL —*of *S. italica*) and heat stress response (*17.9 kDa class II heat shock protein—HSP17.9—*of *L. arundinaceum*), apart from the *phytoene desaturase* (*PDS*) gene used as endogenous marker (Table 1 and references therein). In most of the cases the cited publications discuss the targeted mutagenesis method and results obtained, but the phenotypic characterization of the mutants is limited and far away from field trials. Interestingly, not only classical CRISPR-Cas9 system has been used, but also CRISPR-Cas12a in the case of *L. arundinaceum* (Zhang et al., 2021) and CRISPR-Cas9_Trex2 in the case of *S. viridis* (Weiss et al., 2020).

The toolkit of CRISPR-Cas applications has expanded to around twenty different techniques that allow diverse targeted modifications in the genome (Villalobos-López et al., 2022; Capdeville et al., 2023). On the one hand, Cas enzymes from different bacteria have been characterized and adopted for use. That is the case for Cas12a (former Cpf1), an enzyme from the *Lachnospiraceae bacterium* ND2006 that cuts DNA strands distal from the sequence recognized by the nuclease (the PAM site), generating 4-5 nucleotide overhangs that enable an easy insertion of donor DNA sequences (Zetsche et al., 2015; Moreno-Mateos et al., 2017). Other modifications of the CRISPR-Cas9 system imply the co-expression or the fusion of different proteins to the Cas9 nuclease, in its original or mutated versions. CRISPR-Cas9_Trex2, for example, has the Trex2 exonuclease co-expressed with Cas9 for increasing the mutation efficiency (Čermák et al., 2017; Weiss et al., 2020). Importantly, an enzymatically inactive variant of Cas9, called "dead Cas9" (dCas9) that maintains its specific DNA binding ability, can be fused to transcription activators or repressors to regulate transcriptional levels of endogenous genes (Ding et al., 2022). Therefore, CRISPR-Cas tools are not only meant to inactivate genes and create loss-of-function mutants, but also gain-of-function mutants can be obtained. In addition, thanks to the Super Nova Tag (SunTag) system, the transcriptional regulation can be potentiated. The SunTag contains peptide repeats that bind several transcription factors for cooperatively activating a target gene (Tanenbaum et al., 2014). Moreover, a gene of interest may also be up- or downregulated epigenetically. For instance, CRISPR-dCas9 linked to DRM methyltransferase catalytic domain targets methylation to specific loci and thereby inactivates the target gene (Papikian et al., 2019).

An alternative way of inducing a change in the levels of expression of a gene is altering its promoter sequence. In fact, the promoter can be even swapped by another one that ensures e.g., higher levels of expression in a ubiquitous manner. Using CRISPR-Cas9 such a substitution is possible, as shown for the *auxin-regulated gene involved in organ size 8* (*ARGOS8*) gene in maize, whose overexpression was associated with improved grain yield under field drought stress conditions (Shi et al., 2017).

It should be pointed out that yield and stress resistances are among the most difficult polygenic traits to improve through genetic engineering, but examples as the former one give hope that it can be achieved by CRISPR-Cas. Another example is the knockout *via* CRISPR-Cas9 of the main effect gene *type-B response regulator 22* (*OsRR22*) that controls salt tolerance in rice. Obtained plants showed salt tolerance in growth chambers and no difference in agronomic traits compared to wild type plants in field trials under normal growth conditions (Zhang et al., 2019; Han et al., 2022a).

As explained in section 2, abiotic stress responses are complex, linked to different metabolic pathways and the genes involved in those mechanisms are mainly pleiotropic. Fishing out a specific key player, a master gene to be mutated, could be possible in some cases and it is worth trying. Since genome editing in grasses is in its early stages (Table 1), we selected specific genes related to the four abiotic stresses discussed in this review and figured out if those target genes would need to be overexpressed or downregulated to gain tolerance to specific stresses. The suggested genes can be found in Table 2. If a candidate gene was found in forage grasses or at least in a Poaceae species, that species was selected, but this was not possible in all cases. As shown in Table 2, there are genes that are related to more than one stress response. For simplicity, it is not shown that, e.g., *DHN11* seems to be also involved in cold and drought stresses and *COR410* appears to be related to drought stress as well.

Section 2 mentioned that plants detect an increase in temperature (in the soil or air) when the structure and fluidity of their cell membranes change. Heat stress tends to make membranes more fluid (Niu and Xiang, 2018), which activates pathways through heat sensors like the CNGCs. In theory, an increased expression of stress receptors can lead to an improved response to stress. Consequently, the genes involved in the heat stress response signaling pathway can be upregulated by overexpressing a heat sensor coding gene. In *A. thaliana*, an overexpression of the *SYTA* gene resulted in higher

TABLE 2 Target genes for improvement of abiotic stress tolerance.

Abiotic stress	Target gene	Species	Stress Role	Proposed Strategy	Publication
Heat	*OspsbA*	*Oryza sativa*	Response	Upregulate	(Chen et al., 2020)
	LaHsfA2c	*Lolium arundinaceum**	Response		(Wang et al., 2017)
	OsCNGC14 OsCNGC16	*Oryza sativa*	Sensing		(Cui et al., 2020)
	SlMAPK3	*Solanum lycopersicum*	Response	Downregulate	(Yu et al., 2019)
	OsPYL1/4/6	*Oryza sativa*	Response		(Miao et al., 2018)
	SlPHYA SlPHYB1B2	*Solanum lycopersicum*	Response		(Abdellatif et al., 2022)
Cold	*EnCOR410*	*Elymus nutans*	Response	Upregulate	(Fu et al., 2016)
	AcSnRK2.11	*Agropyron cristatum*	Response		(Xiang et al., 2020)
	OsCOLD1	*Oryza sativa*	Sensing		(Ma et al., 2015)
	OsMYB30	*Oryza sativa*	Response	Downregulate	(Zeng et al., 2020)
	AtEGR2	*Arabidopsis thaliana*	Response		(Ding et al., 2019a)
	AtCRPK1	*Arabidopsis thaliana*	Response		(Liu et al., 2017c)
Drought	*CdDHN4*	*Cynodon dactylon*	Response	Upregulate	(Lv et al., 2017)
	OsSYT-5	*Oryza sativa*	Sensing		(Shanmugam et al., 2021)
	AcSnRK2.11	*Agropyron cristatum*	Response		(Xiang et al., 2020)
	OsDST	*Oryza sativa*	Response	Downregulate	(Santosh Kumar et al., 2020)
	TaSal1	*Triticum aestivum*	Response		(Abdallah et al., 2022)
	HvCBP20	*Hordeum vulgare*	Response		(Daszkowska-Golec et al., 2020)
Salinity	*ZmDHN11*	*Zea mays*	Response	Upregulate	(Ju et al., 2021)
	AcSnRK2.11	*Agropyron cristatum*	Response		(Xiang et al., 2020)
	OsOSCA1.4	*Oryza sativa*	Sensing		(Zhai et al., 2020)
	OsbHLH024	*Oryza sativa*	Response	Downregulate	(Alam et al., 2022)
	HvITPK1	*Hordeum vulgare*	Response		(Vlčko and Ohnoutková, 2020)
	OsRR22	*Oryza sativa*	Response		(Zhang et al., 2019)

*Festuca arundinacea.

germination and seedlings survival rates than in wild-type and knockout lines after heat stress exposition. Moreover, the overexpression plants presented higher expression of both HSPs and HSFs, together with lower levels of membrane lipid peroxidation than in non-overexpression lines (Yan et al., 2017). All these changes provide evidence that upregulating a heat stress sensor can improve the stress tolerance of a plant. Therefore, overexpressing a similar gene in grasses, like a homologous of rice *OsCNGC14* or *OsCNGC16* gene, could result in forage species with higher tolerance to heat stress. A similar approach can be followed by upregulating proteins present in plants in a basal state that are involved in the responses to abiotic pressures (Figure 1). Kinase proteins are suitable for this goal since they are involved in most stress response pathways, regulating posttranslational modifications of other proteins as a response to both abiotic and biotic stress (Damaris and Yang, 2021). Therefore, overexpressing a gene from the SnRK2 family, a group of kinases specific to plants that have been shown to play important roles in abiotic stress regulation is an adequate approach (Zhang et al., 2016). The heterologous overexpression of the gene *TaSnRK2.3* from wheat in Arabidopsis produced plants that had higher tolerance to drought conditions (Tian et al., 2013). Similarly, another study was able to overexpress the *AcSnRK2.11* gene from *Agropyron cristatum* (L.) Gaertn., a forage grass species, in *Nicotiana tabacum* L. The overexpression plants had significantly higher survival rates than the wild-type ones after recovery periods from cold stress and presented significantly upregulated patterns of abiotic stress-related genes like dehydrins. Possibly, upregulation of these protein kinases could provide drought, cold and salinity stress tolerance to forage grasses plants.

On the other hand, negative regulators of abiotic stress responses are also suitable targets for abiotic stress tolerance improvement by downregulating them *via* genome editing (Figure 1). Possible candidates for downregulation could be enzymes that degrade signaling molecules involved in stress response, like for example the inositol phosphatases (Jia et al., 2019). As previously mentioned in this review, the phosphatase SAL1 negatively regulates plants' response to drought (Chan et al., 2016). Using the CRISPR-Cas9 system, scientists have already generated *Tasal1* knockout mutant

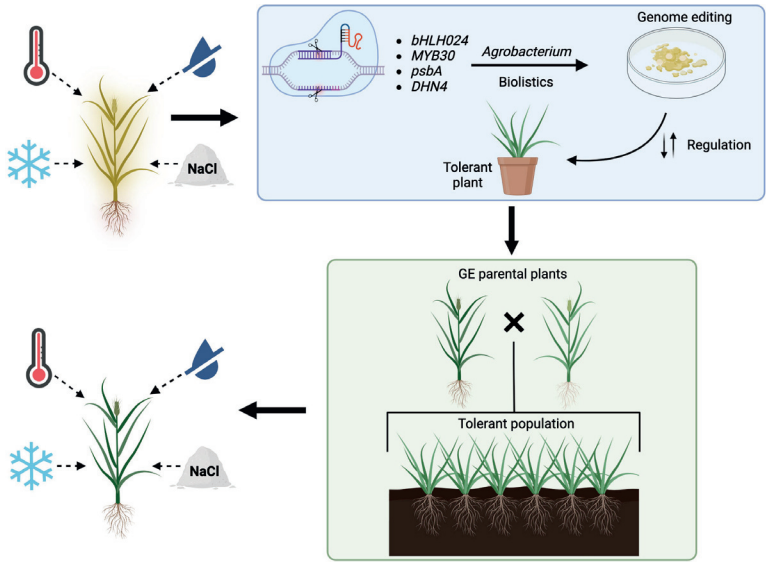

FIGURE 1
Proposed strategy for the improvement of abiotic stress tolerance in forage grasses using genome editing. Four abiotic stresses (heat, low temperature, drought, salinity) hinder the overall wellbeing of a non-tolerant grass (plant shown in yellow). Using the CRISPR-Cas system, different genes can be targeted. Agrobacterium-mediated transformation or biolistics are suitable delivery methods of the CRISPR-Cas+gRNAs complex for in-vitro culture modifications that lead towards the generation of abiotic stress tolerant plant (blue rectangle). Once tolerant parental plants are obtained (GE, gene editing), these can be crossed to produce a population able to overcome the effects of abiotic stress (green rectangle). The green plant on the bottom left represents a tolerant grass.

wheat with fewer and smaller stomata, that germinate and grow better under drought conditions (Abdallah et al., 2022). Likewise, modifying the expression of transcription factors related to abiotic stress is another alternative for producing tolerant plants. The transcription factors of the basic helix-loop-helix (bHLH) family have been shown to participate in abiotic stress regulation in different plant species (Guo et al., 2021). In rice, the *OsbHLH024* gene seems to negatively regulate salinity tolerance. This was demonstrated by generating knockout plants using the CRISPR-Cas9 system. The mutated plants had an increased salinity tolerance when compared to the wild-type ones. Additionally, the knockout lines presented a reduced accumulation of sodium ions and ROS, but higher concentrations of potassium ions than the control plants. Finally, the expression of genes encoding ion transporter was upregulated in the knockout plants in comparison to the wild-type ones (Alam et al., 2022). All these variations suggest that the downregulation of homologues of the *OsbHLH024* gene in grasses could provide them with salinity stress tolerance.

During the last years, innovative ways of inserting specific targeted mutations based on CRISPR-Cas have been developed, e.g., base- and prime editing and for now, some technical problems need to be overcome when applied to plants (Anzalone et al., 2020; Zhu et al., 2020; Hua et al., 2022). In these cases, the Cas9 nuclease is mutated in such a way that it acts as nickase, cutting only one strand of the targeted DNA. These strategies and the activation of homology-directed repair (HDR) instead of Non-Homologous End Joining (NHEJ), makes it possible to produce a wide range of mutations from single nucleotide changes and small indels to increasingly larger insertions and deletions, replacements or even to generate chromosomal rearrangements (Puchta et al., 2022; Villalobos-López et al., 2022).

The possibilities to induce targeted changes with CRISPR-Cas in the genome of crops, and specifically in forage grasses, are immense, not to mention the speed of obtaining the desired traits compared to conventional breeding techniques. In addition, genome editing can be easily multiplexed for targeting different sequences at one shot. Depending on the specific trait and species, there can be bottlenecks to be removed like specific ways of transforming a plant or availability and annotation quality of the reference genome. These obstacles are thought to be solved with technical advances, however in the case of grasses, important biological features need to be taken into consideration when aiming to combine genome editing with a breeding program. These challenges are elaborated in section 5. Here we briefly mention that also reproductive characteristics of grasses can be changed with genome editing.

Forage grasses have a strong gametophytic self-incompatibility (SI) system that makes inbreeding almost impossible. The two multi-allelic *S* and *Z* genes have since long been known to govern SI in grasses (Lundqvist, 1955; Cornish et al., 1979), and recently it was shown that two *DUF247* genes are behind the *S* and *Z* loci (Manzanares et al., 2016; Herridge et al., 2022). With the sequences and molecular function of these genes known, they would be an obvious target for generating self-fertile knockout lines by genome editing. A similar approach has been used to develop self-compatibility in potato (Ye et al., 2018).

To obtain male sterile lines is also of importance in the case of forage grasses. The way has been paved by research in maize, where genes *male sterility 1* (*Ms1*) and *Ms45* have been targeted by CRISPR-Cas9 and male-sterile wheat lines for hybrid seed production have been obtained (Singh et al., 2018; Okada et al., 2019).

Fully homozygous doubled haploid lines can be generated by artificially inducing haploids with a knockout of *MTL* gene, as it has been done already in *S. italica* (Table 1) (Cheng et al., 2021).

Finally, apomixis is present in several grass species, e.g., *Poa pratensis*, a species used both in lawns, pastures, and leys. Inducing apomixis in other forage grasses would be of importance for genetic fixation of hybrid vigor of parental line. Some steps towards achieving this aim have been taken already in rice. Mutations using CRISPR-Cas of several genes related to the abolishment of meiotic steps produced clonal diploid gametes. Then, parthenogenesis was induced by ectopic expression in the egg cell of *BABY BOOM1* and clonal progeny was obtained (Khanday et al., 2019; Zhu et al., 2020).

4 Genome editing versus traditional genetic modifications

Genetic variation is fundamental to crop improvement. Modern plant breeding started in the late 19th century with the advent of cross-breeding which still is the backbone of most plant breeding efforts (Hickey et al., 2019; Gao, 2021). After the discovery that physical and chemical factors can lead to heritable changes in genetic material, random mutagenesis became a valuable tool for plant breeding to increase genetic diversity and to develop specific traits. With the discovery of recombinant DNA technology in the 1970s, the development of new combinations of genetic elements by splicing genes and regulatory elements from different species became possible. The discovery of *Agrobacterium*-mediated transformation enabled scientists to introduce these novel combinations of genes into plant genomes to produce new traits (Gao, 2021). While the introduction of transgenes into plant genomes has contributed enormously to the understanding of gene functions in plants, the commercial applications have been limited to mostly herbicide tolerance and insect resistance, which provide obvious advantages for farmers, but little direct, tangible benefits for consumers in developed countries. Only a few commercial applications of transgenic plants with improved yield and abiotic stress resistance are known. Wheat expressing the sunflower transcription factor HomeoBox 4 (HaHB4) has been shown to provide improved water use efficiency resulting in higher grain production (González et al., 2019). Wheat HB4 marketed by the company Bioceres Crop Solutions has been authorized for food and feed uses in a number of countries, such as Argentina, Australia, Brazil and United States, but its cultivation is approved only in Argentina (Argentina First to market with drought-resistant GM wheat, 2021; HB4 Wheat| GM Approval Database-ISAAA.org, n.d.). Maize MON87403 contains the *ARABIDOPSIS THALIANA HOMEOBOX 17* (*ATHB17*) gene from *A. thaliana* encoding a transcription factor of the HD-Zip II family with reported increase in ear biomass at the early reproductive phase (Rice et al., 2014), which may provide an opportunity for increased grain yield under field conditions (Leibman et al., 2014). Maize MON87460 expresses the *Bacillus subtilis* cold shock protein B (CspB) resulting in increased grain yield under drought conditions (Nemali et al., 2015). Both GMO events have been assessed by the European Food Safety Authority (EFSA) (EFSA Panel on Genetically Modified Organisms (GMO) et al., 2012b; EFSA Panel on Genetically Modified Organisms (GMO) et al., 2018), and MON87460 was authorized for food and feed uses in the EU. Transformation techniques have been developed for most of the economically important forage and turf grass (Wang and Ge, 2006), however, very few transgenic forage grasses have been registered for commercial cultivation. The ISAAA GMO approval database lists only one transgenic event in creeping bentgrass (*Agrostis stolonifera* L.) with tolerance to glyphosate (ASR368) (Creeping Bentgrass (Agrostis stolonifera) GM Events | GM Approval Database - ISAAA.org, n.d.).

Even though commercial cultivation of GM crops has brought clear benefits to farmers and more indirect benefits to environment through reduced land and pesticides use (Brookes, 2019; Brookes, 2022), cultivation and use of transgenic plants for food and feed have been controversial in many regions of the world, and especially in Europe. Agronomic, environmental, human health, social and economic effects of transgenic crops have been comprehensively reviewed by the US National Academies of Sciences in 2016 (National Academies of Sciences, Engineering, and Medicine et al., 2016).

Genome editing became possible with advances in protein engineering which allowed production of site-directed nucleases (SDNs), such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Shukla et al., 2009; Urnov et al., 2010). As outlined in section 3, genome editing has several advantages over the transgenic techniques including precision, lower number of off-target effects, more streamlined production, multiplex possibility, as well as potential for modification of many more different traits. A few examples include lower gluten content in wheat through simultaneous editing of alpha-gliadin genes (Sánchez-León et al., 2018), increased production of gamma-aminobutyric acid in tomato (Li et al., 2018) or increased accumulation of provitamin D3 in tomato (Li et al., 2022). The maize with an increased expression of *ARGOS8* gene, as detailed in section 3, contained no exogenous DNA sequences, thus, theoretically, it could be exempt from GMO regulation depending on country-specific policies.

The increased precision, low off-target potential and the absence of exogenous DNA in some of the genome-edited plants suggested that genome editing would not be regulated similarly to GMOs. For example, in Japan Sanatech Seed has commercialized high gamma-aminobutyric acid tomato (Waltz, 2021). In the EU, however, the Court of Justice of the European Union (CJEU, case C-528/16) ruled that organisms resulting from mutagenesis techniques in legal aspects are GMOs and are subject to the regulations laid down by the Directive 2001/18/EC. This applies to mutagenesis techniques introduced since 2001, when the GMO Directive was adopted. Site-directed nucleases can modify plant genomes according to three scenarios, SDN-1, SDN-2 and SDN-3 (EFSA Panel on Genetically Modified Organisms (GMO) et al., 2012a), where only SDN-3 scenario results in transgenic plants, while under SDN-1 and SDN-2 scenarios no exogenous DNA is inserted into the genome.

However, under the CJEU ruling, also the SDN-1 and SDN-2 techniques, including CRISPR-Cas fall under the GMO Directive, while chemical and radiation random mutagenesis remains exempt according to Annex IB of the Directive 2001/18/EC. The ruling provoked a strong response from both academia and biotech industry, which stressed that from a scientific point of view the application of GMO Directive to products created by a much more precise technique than random mutagenesis and transgenesis results in a disproportionate regulatory burden (Purnhagen et al., 2018; Urnov et al., 2018; Christiansen et al., 2019; Wasmer, 2019; Schulman et al., 2020). It was also noted that this ruling leads to a situation when two identical products with the same mutation resulting in, e.g., herbicide tolerance trait could be regulated in different ways. In addition, it would create an unsustainable situation with detection, since no technology can determine the origin of simple mutations, such as single nucleotide polymorphisms. Consequently, reliable detection methods for SDN-1 and SDN-2 products are problematic (European Network of GMO Laboratories (ENGL), 2019). This legal uncertainty makes genome-editing research in the EU less appealing, as seeking regulatory approval for gene-edited products would involve the same cumbersome procedure as for GMOs. So far there are no applications for regulatory approval involving gene-editing, although a few applications for authorization of products obtained with CRISPR-Cas9 in SDN-3 scenario, e.g., maize DP-915635-4 have been submitted to member states and are currently under review by EFSA (Maize DP-915635-4, n.d.).

According to the EU (Council Decision (EU) 2019/1904), the European Commission (EC) conducted a study involving input from the Member States and different stakeholders regarding the status of new genomic techniques (NGTs) including genome editing. Within this framework, the EC mandated EFSA to issue a scientific opinion on the risk assessment of plants produced by the SDN-1, SDN-2, and oligonucleotide-directed mutagenesis techniques. EFSA has assessed the safety of plants developed using SDN-1 and SDN-2 techniques and did not identify new hazards specifically linked to these techniques compared to both SDN-3 and conventional breeding. In addition, EFSA concluded that the existing Guidance for risk assessment of food and feed from GM plants and the Guidance on the environmental risk assessment of genetically modified plants are sufficient, but only partially applicable, to plants generated *via* SDN-1 and SDN-2 (EFSA Panel on Genetically Modified Organisms (GMO) et al., 2020; Rostoks, 2021). As part of the ongoing effort to update the EU GMO legislation upon EC request, EFSA recently produced an updated scientific opinion on cisgenesis and intragenesis (EFSA Panel on Genetically Modified Organisms (GMO) et al., 2022b). The EFSA scientific opinion concluded that no new risks were identified in cisgenic and intragenic plants obtained with NGTs, as compared with those already considered for plants obtained with conventional breeding and established genomic techniques, although only limited information on such plants was available. EFSA determined that the use of NGTs reduces the risks associated with potential unintended modifications of the host genome resulting in fewer requirements for the assessment of cisgenic and intragenic plants, due to site-specific integration of the added genetic material. However, there was no legal necessity to overhaul the GMO legislation, since the EFSA concluded that the current guidelines were partially applicable and sufficient. Importantly, the data requirements could be reduced on a case-by-case basis for the risk assessment of cisgenic or intragenic plants obtained through NGTs. While cisgenesis and intragenesis is just one of the possible approaches for forage grass breeding, EFSA also recently issued a statement on criteria for risk assessment of plants produced by targeted mutagenesis, cisgenesis and intragenesis (EFSA Panel on Genetically Modified Organisms (GMO) et al., 2022a). These criteria could be used by policy makers to design a more flexible and proportionate risk assessment framework for gene edited plants. Recently, several regulatory options have been proposed (Bratlie et al., 2019; Kearns et al., 2021; Gould et al., 2022). They range from maintaining the *status quo* (full risk assessment of genome edited organisms as GMOs) to product-based regulation or regulation based on the presence/absence of foreign DNA in the genome. These two options would be preferable for commercial deployment of genome edited crops, but they would require substantial reexamination of GMO Directive and authorization procedure. The EC is expected to present a new policy and/or legal proposal by the second quarter of 2023. Meanwhile, other jurisdictions around the world have already developed legal framework for genome edited plants, e.g., under Argentina NBT Resolution N° 21/2021, if a product (plant, animal or microorganism) does not have a new combination of genetic material, the product is non-GM and considered as conventional product (Goberna et al., 2022). Different regulatory approaches are summarized in a recent review (Entine et al., 2021).

Interestingly, the "EU GMO database of Deliberate Release into the environment of plants GMOs for any other purposes than placing on the market (experimental releases)" lists over 900 applications for field trials registered by the Member States since 2002 (Experimental releases of GM Plants, n.d.). Among those there is only one application for field trial of high fructan transgenic ryegrass in 2006, and there are no applications for field trials of genome edited forage grasses, although at least 14 field trials of plants edited with CRISPR-Cas9 have been authorized.

In conclusion, while there are a few basic studies on gene function in forage grasses using genome editing technique as described in section 3 of this review, these are yet to see commercial application. The main limiting factor for the investment in research and development of genome edited forage grasses is probably the regulatory uncertainty, especially in the EU. Although edited plants without foreign DNA in the genome are expected to receive the least amount of regulatory scrutiny, they are also less prone to show major changes in relevant traits. This is because gene knockouts or simple gene edits are unlikely to result in complex phenotypes, such as enhanced abiotic stress tolerance, higher yield or improved nutritional composition, especially considering the genetic complexity that has hindered progress in characterization of the genes underlying such traits in forage grasses. Nevertheless, as recent years have witnessed a dynamic development of genome editing tools and genotype-independent transformation approaches along with increasing genomic resources, the manipulation of plant responses may become possible to overcome abiotic stresses when combining modern techniques and good breeding management strategies.

5 Breeding grasses in the genome editing era

Forage grasses are outbreeding species and highly heterozygous due to the strong gametophytic SI system. Inbred line development is thus very difficult with strong inbreeding depression as a result. Therefore, cultivars of forage grasses are usually synthetic populations (Posselt, 2010). Forage grass breeders usually start by phenotypic selection of superior candidate genotypes for traits with high heritability, e.g., heading date and disease resistance, among a large number of spaced plants. However, forage grasses are sown in swards and because yield and other traits will be affected by competition in the swards, such traits cannot be selected on single spaced plants. The candidate genotypes are therefore put in some form of progeny testing system, e.g., polycross to produce half-sib (HS) families or bi-parental crosses producing full-sib (FS) families, and selection for yield and forage quality traits are based the performance of such families in swards (genotypic selection). Synthetic populations/cultivars are constructed by crossing the best genotypes based on their performance in the progeny test or by mixing HS or FS families. The synthetic populations are further multiplied to obtain enough seed for establishing sward plots for testing in multi-location-year trials before the best candidate cultivars are being submitted to official variety testing. A typical breeding cycle will take 10-15 years before synthetic cultivars are available for farmers. With the advent of high-throughput molecular markers, whole-genome sequences, and genomic selection methods, the breeding cycle can be shortened (Rognli et al., 2021; Barre et al., 2022). Specifically, if genome editing is used for specific reproductive traits, like breaking down self-incompatibility, the forage grass breeding cycle could be shortened according to us by 4-5 years.

The success of a breeding program is very much dependent on the genetic variation present in the initial breeding material. Many agronomically important traits, like yield and adaptability to biotic and abiotic stresses, have been partly fixed within elite germplasm, however, they still exhibit large genetic variation and are thus of primary importance in breeding programs (Meyer et al., 2012; Swinnen et al., 2016). This variation might be employed for future improvements of crop productivity and tolerance to stress; however, landraces, closely related species and wild relatives can offer much wider and unexploited germplasm resources (Jonavičienė et al., 2009; Brozynska et al., 2016). Extensive studies of perennial ryegrass diversity among modern European cultivars revealed that modern cultivars are mostly related to ecotypes from north-western Europe (Blanco-Pastor et al., 2019), while most of the natural genetic variation remains unexploited. Later studies on the genetic structure of geographically diverse perennial ryegrass collection supported these findings and in addition showed that latitude was a prominent force shaping the diversity of wild-growing perennial ryegrass populations (Jaškūnė et al., 2020). Furthermore, the ecotypes exhibit biomass and seed yielding potential similar to cultivars (Bachmann-Pfabe et al., 2018; Jaškūnė et al., 2022b), suggesting that ecotypes could serve as valuable trait donors in breeding programs. Field testing of many *L. perenne* ecotypes and cultivars at several Nordic and Baltic locations identified tetraploid Baltic breeding lines and diploid ecotypes from Eastern Europe as being most winter hardy with stable performances across environments (Gylstrøm, 2020). None of the cultivars were among the most stable entries, and diploid ecotypes displayed a larger variation in heading date, regrowth, and winter survival than the cultivars. Thus, there is ample genetic variation still to be exploited within the genetic resources of perennial ryegrass. Induced polyploidization is also widely exploited in forage crop breeding as one of unconventional techniques to develop new superior yielding and abiotic stress tolerant breeding material (Akinroluyo et al., 2019; Akinroluyo et al., 2020; Rauf et al., 2021).

To utilize transgenes or gene-edits in grass breeding, first, efficient methods for introduction and regeneration *in vitro* need to be available in a range of independent genotypes. In principle, introgression of new genes can either be introduced into the parental clones of already existing varieties (variety-parent approach) or transferred into a new base population (population approach) (Posselt, 2010). Repeated backcrossing and an efficient selection system is needed to bring transgenes/gene-edits to homozygosity in the parental clones. A side-effect of this could be increased inbreeding depression due to linkage drags creating longer homozygous chromosomal segments. Traditional random insertion of transgenes in several genotypes that are intercrossed to construct synthetic cultivars is problematic due to the presence of multiple insertion sites, silencing and variable expression levels. The availability of complete genome sequences also of forage grass species, notably *L. perenne* (Nagy et al., 2022), and genome editing technologies, makes it possible to induce precise genome alterations. This will make it easier to develop synthetic cultivars of outbreeding crops like forage grasses with stable expression of genetic modifications.

Integration of transgenic traits in perennial grasses and the challenges associated with deployment and management of transgenic cultivars has been discussed by Badenhorst and colleagues as well as by Smith and Spangenberg (Badenhorst et al., 2016; Smith and Spangenberg, 2016). Using gene-drive technologies (Bier, 2022) would in principle be an efficient method for spreading gene-edits through breeding populations of grasses. However, the risk of gene flow between cultivars and to feral populations is high and would probably preclude practical use of such technologies.

A pertinent question is what the most important targets for genetic engineering in forage grasses would be. Genetic gain for yield has been modest due to the long breeding cycles and extensive field testing (Sampoux et al., 2011; McDonagh et al., 2016). The potential heterosis is only partially exploited in synthetic cultivars, and it is expected that great yield increased could be achieved if F1 hybrids, which has been very successfully exploited in maize, could be developed (Herridge et al., 2020). Self-incompatibility, inbreeding depression, and the lack of male-sterile lines for making hybrids are major obstacles for developing F1 hybrids. Inbreeding depression needs to be tackled to implement self-fertile lines in forage breeding programs. By generating a large number of self-fertile plants with diverse genetic backgrounds by gene-editing, and selecting genotypes with good seed set, the prospects of developing inbred lines in forage grasses have never been better. These lines could be used for F1 hybrid production and would also be very useful for functional studies. Other methods for capturing heterosis would be the development of facultative apomixis. The evolution of apomixis in natural populations and the challenges of utilizing apomixis in breeding has been reviewed recently (Hojsgaard and Hörandl, 2019).

6 Conclusion

In the current review, we focus on possible improvements of abiotic stress tolerance in forage grasses using new genome editing tools. The potential impact of climate change is described in relation to forage grass tolerance to four important abiotic stresses, such as heat, low temperature, drought and salinity. We propose approaches for editing the genome of grasses to regulate stress responses. Furthermore, we discuss the latest developments in the regulatory framework for genome editing, especially with regard to the EU, and identify factors affecting the application of genome editing techniques for the improvement of grasses. Finally, we address breeding strategies specific to the reproductive biology of forage grasses and identify how genome editing could be used to facilitate breeding and achieve food security in a sustainable way. In conclusion, we describe pathways for developing abiotic stress tolerance in forage grasses under climate change using genome editing technologies, provided that an appropriate legal framework is developed.

Author contributions

Conceptualization, FS-S, CS. writing—manuscript preparation, FS-S, NR, KJ, MS, OAR, MRK, GS and CS. writing—review and editing, FS-S and CS. funding acquisition, KJ, NR, OAR and CS. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Isolation and transformation of perennial ryegrass (*Lolium perenne* L.) protoplasts for the *in vivo* assessment of guide RNAs editing efficiency

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Protoplasts are broadly used to perform different cellular and genetic assays. Transformation of protoplasts requires isolation methods that generate a large number of intact cells suitable for downstream applications. *Lolium perenne* L. is an important forage grass species for which gene editing techniques are in their early stages. Using protoplasts has previously been reported as a suitable approach to test the genome editing efficiency of guide RNAs in important grass species like wheat and rice. This approach can speed up and increase the chances of generating edited plants, especially when working with species for which stable transformation methods have not been established yet. Testing two different approaches regarding the processing of *L. perenne* L. tillers showed that using a blender for disintegrating the tissue was easier and faster than cutting the tillers with a razor blade. Conversely, the more classical strategy (cutting with a razor) provided a higher number of viable protoplasts. The use of an enzyme solution containing 2% cellulase during 8 h was shown to be the best experimental condition for protoplast isolation. The addition of a sucrose cushion improved the purification of alive cells, which were then positively transformed with guide RNA encoding vectors using polyethylene glycol. The presence of indels induced by these vectors was then confirmed through decomposition-based analysis of their sequenced genomic DNA. These results demonstrated the suitability of using protoplasts for the *in vivo* assessment of guide RNAs editing efficiency.

KEYWORDS

CRISPR-Cas, gene editing, guide RNA, *Lolium perenne* L., PEG, perennial ryegrass, polyethylene glycol, protoplasts

1 Introduction

The rapidly growing population demands safe food and feed and thus the need for the development of high-yielding crops adaptable to future climate conditions. In temperate regions, climate change may provide the opportunity to expand the list of cultivated forage crops for livestock farming, but on the other hand it may compromise already cultivated species (McEvoy et al., 2011; Grinberg et al., 2016; Kemešytė et al., 2017, Kemešytė et al., 2023). Perennial ryegrass (*Lolium perenne* L.) is the predominant forage grass species in livestock farming (Humphreys et al., 2010), due to its quick establishment, robust regrowth, and high nutritional value for ruminants (Wilkins, 1991). However, perennial ryegrass performs poorly under adverse environmental conditions compared to other cool-season forage grass species, compromising its cultivation of it in northern-eastern regions of Europe (Kemešytė et al., 2017; Cyriac et al., 2018). Thus, it is of vital importance to develop crops with high, stable, and good quality yields. The development of new cultivars of outcrossing species using traditional breeding techniques is a long and time-consuming process (Pfender, 2009; Sampoux et al., 2011; Sustek-Sánchez et al., 2023).

Moreover, this reproductive system makes *L. perenne* a highly heterogeneous and heterozygous species, which creates an additional challenge when performing genetic studies. Forward-genetics approaches used in crops to identify e.g., abiotic stress resistance genes cannot be easily applied in perennial ryegrass. Genome editing using CRISPR-Cas systems offers an alternative solution for identifying mechanisms underlying stress tolerance and achieving increased frost and drought tolerance of *L. perenne* (Sustek-Sánchez et al., 2023). Nevertheless, when generating edited plants, there is usually a need for the induction, propagation, transformation, and regeneration of calli, making the process time-consuming and complex. Furthermore, in some important species like perennial ryegrass, the generation, transformation, and regeneration of calli has either not been achieved or is in the early stages of methods development with each of the above steps being highly genotype dependent. To date, few publications describe induction of CRISPR-Cas9-targeted mutations in *L. perenne*, all of which used embryogenic calli for either biolistic (Zhang et al., 2020) or *Agrobacterium*-mediated transformation (Grogg et al., 2022; Kumar et al., 2022). The scarcity of available data thoroughly evaluating the impact of each protocol step slows down the development of robust, efficient, and genotype-independent methods for the transformation of *L. perenne*. Screening plasmids containing the Cas9 gene and gRNA arrays for gene editing efficiency prior to their use in calli transformation, makes the process of obtaining edited perennial ryegrass genotypes more time and resource efficient.

The design of guide RNAs (gRNAs) relies generally on bioinformatic tools that predict how efficient and specific a guide is expected to be. However, the efficiency of a gRNA predicted using in silico tools often does not agree with experimental data (Bennett et al., 2020; Konstantakos et al., 2022). This showcases the need for empirically testing the gRNAs *in vivo*, to evaluate their ability to generate indels.

The transformation of protoplasts is an efficient method to perform diverse cellular, molecular, and genetic studies (Eeckhaut et al., 2013; Wu and Hanzawa, 2018). They have also been proved useful in evaluating the efficiency of gRNAs and editing reagents to generate genome-edited plants (Shan et al., 2013; Nadakuduti et al., 2019; Brandt et al., 2020; Yue et al., 2021; Hu and Huang, 2022). Protoplasts can be isolated by millions, which provides an excellent tool for testing genome editing applications. Genomic DNA from transformed protoplasts can be analyzed by sequencing PCR-amplified regions of interest. The application of decomposition-based tools, such as TIDE, allows the identification of edited cells and thus the evaluation of genome editing efficiency (Brinkman et al., 2014).

In this study, we aimed at establishing a reproducible method that would allow *in vivo* evaluation of the editing efficiency of specific gRNAs and transformation vectors using protoplasts. While similar approaches have been used in other grasses, reproducible protocols are lacking for *Lolium perenne*. For this, two different protoplasts isolation methods were studied and compared to determine the best performing technique, yielding a high number of protoplasts whose quality would make them suitable for downstream applications such as polyethylene glycol (PEG)-mediated transformation. Two different genes were used as targets for the evaluation of editing efficiency of binary vectors. *CBP20* is a gene related to cuticular wax accumulation, which has been used to generate drought tolerant barley plants by knocking it out (Daszkowska-Golec et al., 2017, 2020). *CRPK1* is a known negative regulator of proteins resulting from the activation of the ICE-CBF-COR pathway by ABA-dependent responses (Liu et al., 2017; Pashapu et al., 2024).

The DNA of transformed cells can be sequenced and analyzed using decomposition methods to identify and evaluate the presence of indels. This can be used to assess the editing efficiency of reagents such as binary vectors coding for specific gRNAs. In organisms recalcitrant to common transformation methods, such as perennial ryegrass via Agrobacterium-mediated transformation, the described *in vivo* screening can accelerate the generation of edited plants.

2 Material and methods

2.1 Plant material

The plant material was obtained from germinated seeds of the *Lolium perenne* cv. Veja. and from *in vitro* propagated tillers of a freezing sensitive perennial ryegrass genotype (previously characterized in Pashapu et al., 2024). Plant material from cv. Veja was used to test the different parameters of the protoplasts isolation procedure and to perform the transformations aimed at editing the perennial ryegrass *CBP20* gene (*LpCBP20*). Plant material from a freezing sensitive genotype was used for the transformation experiments to edit the *CRPK1* gene of *L. perenne* (*LpCRPK1*).

The seeds were surface sterilized by washing them with 70% ethanol (3 min), commercial bleach (5% sodium hypochlorite, 90 min), followed by autoclaved distilled water (3 to 5 washes of 1 min). After sterilization, the seeds were placed in 6-well plates containing 2 mL per well of autoclaved liquid half-strength Murashige & Skoog media (MS, Duchefa Biochemie) supplemented with 1% (w/v) sucrose (pH adjusted to 5.7). Thereafter, seeds were cold stratified by keeping them at 4 °C for 24h, to induce homogenous germination, and grown for three weeks in a 16 h photoperiod (26 µmol m-2 s-1), at 21 °C and 60% relative humidity in a growth chamber (Sanyo, MLR-351).

Tillers obtained from one germinated seedling, as well as *in vitro* propagated tillers, were used for protoplast isolation. The propagated tillers were grown for three weeks in sterile solid MS media supplemented with 3% (w/v) sucrose, thiamine·HCl (1 mg/L), nicotinic acid (0.5 mg/L), pyridoxine (0.5 mg/L), 6-Benzylaminopurine (2 mg/L), and 0.3% Gelrite (all components from Duchefa Biochemie; pH 5.7) and with the same light, temperature, and humidity conditions as described before. Tillers were moved onto fresh media before protoplast isolation. All plates used for germination or propagation were sealed with Micropore™ tape (3M).

2.2 Isolation of protoplasts

The effect of the following conditions on the efficiency of protoplast isolation was tested: tissue disintegration instead of cutting, cellulase and mannitol concentrations, enzymatic treatment length, and the use of vacuum infiltration.

For the classical approach, approximately 2 g of fresh weight (gFW) of tillers were cross sectioned into 1–2 mm pieces, placed in a 20 cm Petri dish, and treated with enzyme solution (10 mL per gFW). Since the sectioning of tillers is one of the most time-consuming parts of the protoplast isolation procedure, an alternative method for tiller disintegration was tested as follows.

The same amount of *in vitro* culture derived tillers (2 gFW) was disintegrated using a Waring® laboratory blender (Sigma-Aldrich, catalog number Z272221) in a stainless-steel mini container (250 mL) with 50 mL of sterile mannitol solution (0.5 M), applying blending mode "low" and using short pulses. Four different blending conditions were tested: blending was applied by one, three, five, and ten pulses. Disintegrated tissues were collected by filtering through a 40 µm cell strainer (EASYstrainer™, Greiner Bio-One), moved to a Petri dish with 50 mL of enzyme solution (2% cellulase, composition described in Table 1) and incubated for 8 hours. The blending experiments were repeated three times.

When sectioning the tillers, different concentrations (w/v) of cellulase (Onozuka R-10, Duchefa Biochemie) were tested: 1.5, 2.0, 2.5 and 3.0%. The complete formulation of the enzyme solution, which included Macerozyme R-10 (0.75% w/v, Duchefa Biochemie), can be found in Table 1 and is based on previous methods described for the generation of mesophyll protoplasts of perennial ryegrass by Yu et al. (2017) and Davis et al. (2020).

TABLE 1 Composition and storage conditions of the solutions used for protoplast isolation and transformation.

Solution	Composition
Enzyme solution[a]	2% cellulase Onozuka R-10, 0.75% Macerozyme R-10, 10 mM MES, 0.6 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 0.1% BSA. pH adjusted to 5.7 with KOH.
W5[b]	2 mM MES, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl. pH adjusted to 5.7 with NaOH.
W5A[b]	0.5 mM MES, 5 mM glucose, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl. pH adjusted to 5.7 with NaOH.
WI[b]	4 mM MES, 500 mM mannitol, 5 mM KCl. pH adjusted to 5.7 with NaOH.
MMG[b]	4 mM MES, 400 mM mannitol, 15 mM $MgCl_2$. pH adjusted to 5.7 with KOH.

[a]Storage and preparation: Mix MES, KCl, and mannitol, adjust the pH and autoclave. Keep at 4 °C for up to one month. On the day of the experiment, add the enzymes and heat the solution at 55 °C. Add sterile CaCl2 and BSA, when at RT.
[b]Autoclaved and kept at 4 °C for up to one month.

To determine the most suitable digestion length, we incubated the tillers for 8, 12, 16, and 20 h. Four different concentrations of cellulase were tested during these four time points and protoplasts were counted after each of them using fluorescein diacetate (FDA) to determine their viability. 25 µL of FDA solution (5 mg/mL) per mL of protoplast suspension was used, as described by Hu and Huang (2022). Before counting the protoplasts, the suspension treated with enzymes was filtered through a 100 µm cell strainer (EASYstrainer™, Greiner Bio-One) and mounted on a 50 mL tube. The filter was pre-wet with W5 solution (see Table 1), the suspension was transferred to the strainer using cut pipette tips (here and always when handling protoplasts) and 4 mL of W5 solution were added to the filter to further increase the number of protoplasts. Then, the suspension was centrifuged for 5 min, 100 g (with minimal acceleration and deceleration) at 11 °C. The supernatant was discarded, and the pellet was resuspended with an equal volume of W5 washing solution (see Table 1). To determine the best cellulase concentration and enzymatic treatment duration, the experiments were repeated four times.

Once the best cellulase concentration and enzymatic treatment length were established, different molar concentrations of mannitol (0.2, 0.3, 0.5, and 0.6 M) were tested during a plasmolytic pretreatment of tillers. For each mannitol concentration, 20 mL of mannitol solution was added to a Petri dish with non-sectioned tillers, and the plate was incubated for 1 h in the dark at RT with 75 rpm shaking. Cell viability was determined using FDA as previously described. Finally, we tested the use of vacuum for infiltration of the enzyme solution into the protoplasts (Reed and Bargmann, 2021). For vacuum, 71 kPa of pressure was used for 5 minutes, and the treatment was repeated three times by slowly increasing and releasing the pressure. Cell viability was determined using FDA. The mannitol and vacuum experiments were repeated four times each.

A sucrose gradient was used to further improve the quality and quantity of viable cells. After centrifugation of the cells suspended in W5 solution (as described above), the supernatant was discarded,

and the pelleted protoplasts were resuspended in 2 mL of W5A solution (see Table 1). In a 15 mL tube, the suspended cells were layered on top of 4 mL of 21% sucrose (w/v) solution and centrifuged for 10 min, 100 g (acceleration and deceleration set to minimum) at 11 °C (Brandt et al., 2020). The layer containing protoplasts after centrifugation was collected and placed into a round bottom tube. 2–3 mL of suspended protoplasts were retrieved. WI solution (see Table 1) was added in a 2:1 volume ratio of the collected protoplasts suspension. The tubes were left overnight, or for at least 1 hour, in the dark at 4 °C. Thereafter, the protoplasts were pelleted, and the supernatant was discarded and replaced by half of its volume using WI solution.

2.3 Transformation vectors

To evaluate the suitability of the isolated protoplasts for assessing the editing efficiency of CRISPR-Cas9 binary vectors, we designed different gRNAs targeting the first (two gRNAs) and second exon (three gRNAs) of the *LpCBP20* gene, which we used as a testing platform (see Supplementary files 1, 2). The target region was sequenced from cv. Veja and the obtained sequence was used for designing gRNAs with CRISPOR (Concordet and Haeussler, 2018). The latest available *L. perenne* genome assembly was used as a query for the specificity and off-target calculations (GCF_019359855.1) (Frei et al., 2021). gRNAs with high specificity to the targeted region (score equal to or greater than 85) and low prediction of off-targets (below 10 predicted off-targets and 0 off-targets close to a PAM region), were selected.

Two different plasmids were tested: pHSE401/EGFP and pTRANS_HiGRFdGm1. Both plasmids contain fluorescent markers, EGFP and ZsGreen, respectively. The plasmid pHSE401 was a gift from Qi-Jun Chen (Addgene plasmid # 62201) and has a single gRNA cassette (Xing et al., 2014) (Supplementary file 3). Two versions of the plasmid containing a different gRNA (gRNA 196 or gRNA 229) were created following the Golden Gate assembly described in Xing et al. (2014), targeting the second exon of the *LpCBP20* gene. The resultant vectors were named p196 and p229.

Plasmid, pTRANS_HiGRFdGm1, can fit five different guides separated by tRNA repeats. This plasmid was a kind gift from Dr. Sergei Kushnir (Teagasc) and based on vector pTRANS_210d (Addgene plasmid # 91109) described in Čermák et al. (2017), which was used as a backbone. The backbone contains different expression cassettes: one cassette coding for ZsGreen, another cassette coding for a morphogenic regulator (data not published), and a cassette containing an intronic HPTII CDS to provide hygromycin resistance to the transformed plant material (map of the final transformation vector available in Supplementary file 4).

The two guide RNAs previously mentioned (g196 and g229) were used in combination with two others that targeted the first exon (g9 and g22) of the *LpCBP20* gene and an additional guide targeting the second exon of the gene (g220). This vector was labeled as pCBP20_5g (see Supplementary file 4) and was assembled by Dr. Anete Borodušķe (University of Latvia) using the Golden Gate approach into pTRANS_HiGRFdGm1 backbone, following the cloning protocol described by Čermák et al. (2017). The plasmid pMOD_A1110 (Addgene plasmid # 91031) was used as module A and encoded a wheat codon optimized Cas9 nuclease. Plasmid pMOD_B2303 (Addgene plasmid # 91068) was used as module B and encoded a polycistronic cassette suitable for the expression of multiple gRNAs. Plasmid pMOD_C0000 (Addgene plasmid # 91081) was used as module C providing the necessary bases needed for the final assembly of the transformation vector using Golden Gate cloning.

In addition, another *L. perenne* gene, *LpCRPK1*, was targeted using a different plasmid. This plasmid was based on the Level 2 vector EC67907 kindly provided by Wendy Harwood & Cristobal Uauy (Addgene plasmid # 211794) (Lawrenson et al., 2024). The same ZsGreen cassette present in plasmid pTRANS_HiGRFdGm1 was inserted into the Level 2 vector to create the plasmid piCas9_ZsGreen (see Supplementary file 5). This plasmid was used to generate a transformation vector encoding 6 gRNAs targeting three different paralogs of the *LpCRPK1* gene (more information present in Supplementary files 1, 2). The assembly was done according to Lawrenson and colleagues (Lawrenson et al., 2024). Six different Level 1 vectors were used, each one encoding a different gRNA. Plasmid EC70188 (Addgene plasmid # 209449) encoded gRNA 190-1, plasmid EC70196 (Addgene plasmid # 209457) encoded gRNA 190-2, plasmid EC70204 (Addgene plasmid # 209465) encoded gRNA 234-1, plasmid EC70191 (Addgene plasmid # 209452) encoded gRNA 234-2, plasmid EC70199 (Addgene plasmid # 209460) encoded gRNA 232–1 and plasmid EC70207 (Addgene plasmid # 209468) encoded gRNA 232-2. Plasmids pAGM8031 (Addgene plasmid # 48037) and pAGM8079 (Addgene plasmid # 48041) were used as Level M accepters, and plasmids PICH50900 (Addgene plasmid # 48047) and PICH50927 (Addgene plasmid # 48049) were used as Level M linkers. Plasmid piCas9_ZsGreen was used as the Level 2 accepter and plasmid PICH41822 (Addgene plasmid # 48021) was the Level 2 linker.

Two gRNAs were designed for each *LpCRPK1* paralog, targeting the first and second exons. The sequences of each paralog were amplified and sequenced from a perennial ryegrass freezing sensitive genotype (more information available in Pashapu et al. (2024) and used for gRNA design with CRISPOR.

To further estimate the transformation efficiency, empty vectors (i.e., not coding for gRNAs) were used as controls and designated as pEGFP and pDelta for the single gRNA and multi gRNA plasmid, respectively, in comparison to the vectors used to target the *LpCBP20* gene. For the plasmid containing six gRNAs targeting different paralogs of the *LpCRPK1* gene, an empty vector not coding gRNAs was labeled as pCtrl_iCas9 and used as a control.

The sequences of the primers and gRNAs used in this study can be found in Supplementary files 2, 6. All the amplifications performed in this study were done using ThermoFisher's Phusion™ High-Fidelity DNA Polymerase to reduce the chance of polymerase-induced nucleotide variations, as recommended by Tsakirpaloglou et al. (2023). All the transformation vectors,

with and without gRNAs, were checked for correct assembly using an external whole plasmid sequencing service.

2.4 PEG-mediated protoplasts transformation

For the transformation procedure, the volume of protoplasts suspension in MMG (see Table 1) was adjusted to contain 3×10^5 cells. To change the solution from WI, in which they were placed after performing the sucrose cushion, the needed volume for the previously mentioned cell density was placed in a 2 mL tube, which was centrifuged at 100 g (always with acceleration and deceleration set at minimum), RT for 5 min. The supernatant was discarded and replaced by the same volume of MMG solution. Tubes containing 10 µg of either control or gRNA encoding vectors were prepared. The plasmid DNA was obtained by midi-prepping *E. coli* encoding said plasmids using the PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen™) following the instructions described by the manufacturer. The protoplasts suspended in MMG were added to the tubes containing the plasmids and mixed gently. Freshly prepared PEG solution (0.4 g/mL PEG 4000, 0.2 M mannitol, 0.1 M $CaCl_2$) was added to a volume ratio of 1:1 and the suspension was incubated in the dark for 15–20 min, leaving the tubes horizontally after gentle mixing. Following incubation, a wash was performed with W5 solution. The tube was then centrifuged at 100 g and RT for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of WI. The suspension was then passed into a 24-well plate, the wells of which were precoated with 5% BSA. The plate was incubated for 48 h in the dark. This transformation method was adapted from (Brandt et al., 2020). After two days of incubation, samples were collected to calculate the percentage of fluorescent protoplasts and determine the transformation efficiency using an inverted fluorescence microscope (Zeiss Axiovert 200M) and a Neubaer chamber (BLAUBRAND). Genomic DNA was extracted from transformed cells according to Weigel and Glazebrook's protocol with some modifications (Weigel and Glazebrook, 2009). DNA from non-transformed protoplasts was extracted following the same method and used for comparisons.

2.5 Determination of editing efficiency

Genomic DNA from transformed and non-transformed samples was used to perform PCR amplification of the targeted genes *LpCBP20* and *LpCRPK1*. These amplifications were done using ThermoFisher's Phusion™ High-Fidelity DNA Polymerase. To ensure the reliability of the results, the genomic DNA of untransformed protoplasts belonged to the same suspension of cells used for the different transformations.

The generated amplicons were sequenced by Sanger, and the obtained trace data was used to determine the presence of indels in the transformed samples. The editing efficiency of the gRNAs was established using the TIDE tool. Based on what was recommended by the authors of TIDE, the cut-off for the obtained data was: R^2 equal to or greater than 0.9 and indel frequencies considered as statistically significant by TIDE ($P < 0.001$) (Brinkman et al., 2014).

2.6 Statistical analysis and graphical representations

The statistical analysis and graphs were done using GraphPad Prism 10.2.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). A two-way ANOVA test with the Geisser-Greenhouse correction was used to detect statistically significant variations between the different enzymatic treatment time points and cellulase concentrations. Tukey's multiple comparison was used to determine the specific cellulase concentration and enzymatic treatment length that produced the significantly highest number of alive cells. For the blending experiments, pairwise comparisons were assessed by Tukey's multiple comparisons test to analyze the different blending intensities. To evaluate the number of alive cells produced by the different mannitol pretreatments, a one-way ANOVA analysis together with the Geisser-Greenhouse correction was used. To determine the mannitol concentration that yielded the significantly highest number of alive protoplasts, Tukey's multiple comparison was used. A two-tailed unpaired t-test was used to calculate statistical significance for samples with and without vacuum infiltration. For the analysis of the transformation efficiency of the pHSE401/EGFP plasmid, a Welch and Brown-Forsythe ANOVA test was performed together with Dunnett's multiple comparison test. To evaluate the transformation efficiency of the pTRANS_HiGRFdGm1 plasmid coding (pCBP20_5g) and not coding for a gRNA (pDelta), an unpaired t-test was performed. A similar evaluation was done to analyze the transformation efficiency of the plasmid targeting *LpCRPK1* paralogs, by comparing it with the transformation data of a plasmid not encoding gRNAs (pCtrl_iCas9). The editing efficiency of the plasmid with one guide RNA (p196 and p229) was evaluated using an unpaired t-test with Welch's correction. For the vector encoding 5 different guides (pCBP20_5g), the efficiency of the gRNAs was analyzed using a Welch and Brown-Forsythe ANOVA test together with Dunnett's multiple comparison test. The same statistical analysis was used to evaluate the efficiency of the gRNAs part of the plasmid targeting different paralogs of *LpCRPK1* (piCas9_CRPK1).

3 Results

3.1 Protoplast isolation

In this study, we tested two methods for isolation of protoplasts from perennial ryegrass. One method followed the classical cutting

of the tillers in small pieces before the enzymatic treatment, while the other was a novel way of disintegrating the plant tissue using a blender. In both cases, the amount of initial plant tissue was 2 gFW. The experimental design is shown in Figure 1, describing the workflow from collecting the tissue till the outcome of the transformation of the obtained protoplasts.

The yield of isolated protoplasts generated when blending the tillers instead of cutting them can be seen in Figure 2. Using 5 pulses

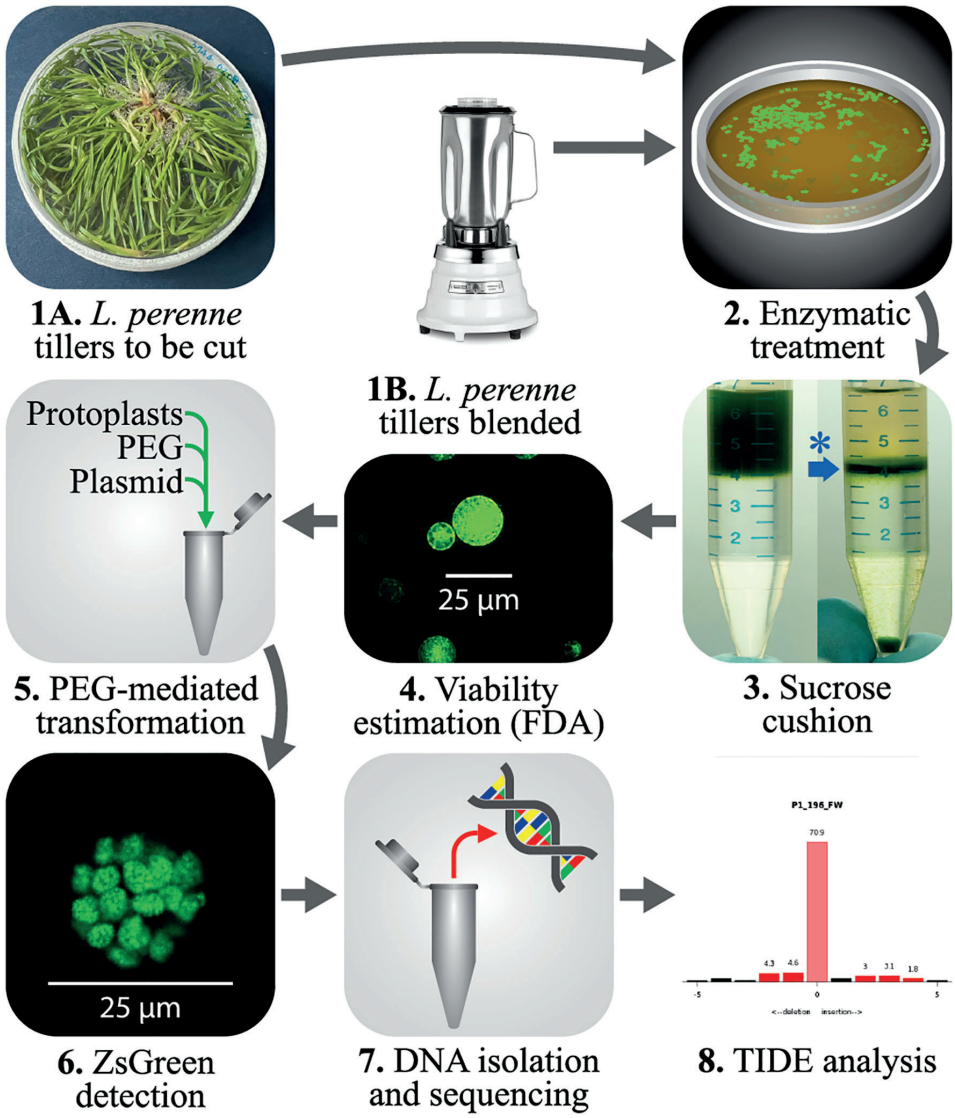

FIGURE 1
Schematic representation of the methods used for isolating perennial ryegrass protoplasts and transforming them for the screening of gRNAs. **1A.** *L. perenne in vitro* grown tillers or seedlings were used as starting material and processed using a razor blade. **1B.** Alternatively, the tillers or seedlings of perennial ryegrass were disintegrated using a Warning laboratory blender. **2.** The tillers were treated with an enzyme solution containing 2% cellulase for 8 h in dark conditions. **3.** A suspension of alive protoplasts was obtained (asterisk and blue arrow) using a sucrose cushion (21%). **4.** The protoplasts suspension was kept overnight at 4 °C, and the cell density and viability were estimated using FDA staining. **5.** 3×10^5 protoplasts were used for PEG-mediated transformation of plasmids encoding 1 or multiple gRNAs. **6.** After 48 h of incubation, the transformation efficiency was calculated by observing green fluorescence in the protoplasts. **7.** DNA from transformed and non-transformed samples was isolated and used for Sanger sequencing. **8.** The presence of indels in the genome of transformed protoplasts was analyzed with TIDE. Image created with Adobe Illustrator 2023.

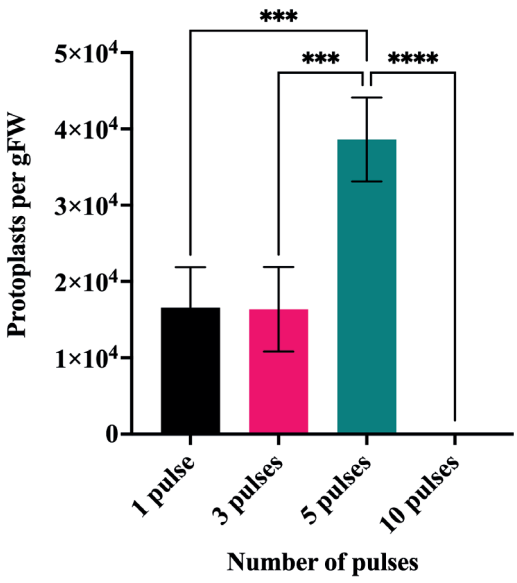

FIGURE 2
The effect of tissue disintegration on the efficiency of protoplast isolation. *In vitro* shoot culture derived from *L. perenne* tillers were disintegrated by blending before enzymatic treatment. Four different blending regimes were compared – disintegration with a single short blending pulse, three pulses, five pulses, and ten pulses. Each bar represents the average number of viable protoplasts per g of fresh weight (gFW) determined by FDA staining of three biological replicates; error bars represent standard deviation. ***P ≤ 0.001 and ****P ≤ 0.0001.

provided a statistically significant higher number of alive cells on average (3.86×10^4 cells per gFW) when compared with the use of 1, 3 or 10 pulses.

In the case of the classical method, cutting tillers with a razor blade, four different incubation times (8, 12, 16, and 20 h) were used to test four different cellulase concentrations (1.5, 2, 2.5, and 3%) present in the enzyme solution. Figure 3 shows the number of counted alive protoplasts after testing these two parameters. The use of 2% cellulase gave the best results in terms of alive cells, independently of the enzymatic incubation time. 8 h incubation produced significantly better results than all other digestion lengths, since after this period the number of protoplasts started to decrease (Figure 3). Therefore, the best yield in terms of alive protoplasts per gFW, approximately 9×10^5, was obtained using an enzyme solution with 2% cellulase to perform the enzymatic digestion for 8 h. The use of a solution with 0.5 M mannitol for pretreatment provided a significantly higher yield, approximately 8×10^5 protoplasts per gFW, than the other mannitol molar concentrations (Figure 4A). Finally, the application of 71 kPa of vacuum pressure gave significantly better results than when no vacuum was used, yielding around 7.7×10^5 alive cells per gFW (Figure 4B). It must be noted though, that even when vacuum was not used, around 6×10^5 living cells per gFW were counted. Increasing vacuum pressure improved protoplast yields also for the blender method, although the difference was not statistically significant (data not shown).

3.2 Protoplast transformation

Fluorescence was detected by microscopy in the protoplasts transformed with both types of plasmids, targeting *LpCBP20* or *LpCRPK1*. This indicated a positive transformation of the cells using PEG. The average number of fluorescent protoplasts with respect to the total counted cells for each of the transformation vectors can be seen in Figure 5. In the case of the single gRNA vectors, no statistically significant differences were observed between the control plasmid (pEGFP) and the plasmids encoding one gRNA (p196and p229 (Figure 5A). The other vectors targeting *LpCBP20* also presented a not statistically significant difference (Figure 5B). Since ZsGreen has proved to be brighter than EGFP (Susič et al., 2014; Cho et al., 2019), the transformation efficiency of the two types of vectors used to edit *LpCBP20* cannot be compared. Moreover, for the plasmids encoding ZsGreen and an intronized Cas9 no statistically significant differences were observed between the control plasmid (pCtrl_iCas9) and the vector encoding 6 gRNAs (piCas9_CRPK1) (Figure 5C). Although vectors encoding an intronized Cas9 appeared to have a higher transformation efficiency than those containing the ZsGreen cassette and a non-intronized nuclease, this difference was not statistically significant.

3.3 Editing efficiency

DNA was extracted from the protoplasts after transformation and Sanger-sequenced. Representative figures showing the amplified PCR products can be seen in Supplementary file 7.

Decomposition-based analysis with the TIDE program was performed and this resulted in the detection of the frequency of indels in the pool of the protoplasts transformed with plasmids coding for one or multiple gRNAs. These results are shown in Figures 6A–C and depict only the events that passed the threshold set for TIDE: indel frequencies with a R^2 equal to or greater than 0.9 and $P < 0.001$. For the plasmids targeting *LpCBP20*, the average editing efficiencies were between 5 and 10%. While no statistically significant difference was observed between the editing efficiency of the plasmids targeting *LpCBP20*, it seemed that in our experiments the plasmids coding for a single gRNA presented on average slightly lower frequencies of indels (7.8%, Figure 6A) than the multiplex plasmid (8.6%, Figure 6B). In the case of the plasmid targeting *LpCRPK1*, the editing efficiency of all the gRNAs was above 10%, except for gRNA 232-2 (Figure 6C). Only samples that met the previously described TIDE threshold for efficiency were plotted and analyzed. Due to this cutoff, the results corresponding to gRNAs 234–1 and 234–2 were excluded from further analysis. No statistically significant differences were observed between the different gRNAs encoded in the vector targeting *LpCRPK1*. Additionally, in one of the analyzed samples two of the targeted paralogs showed the presence of indels in both exons (Figure 6D).

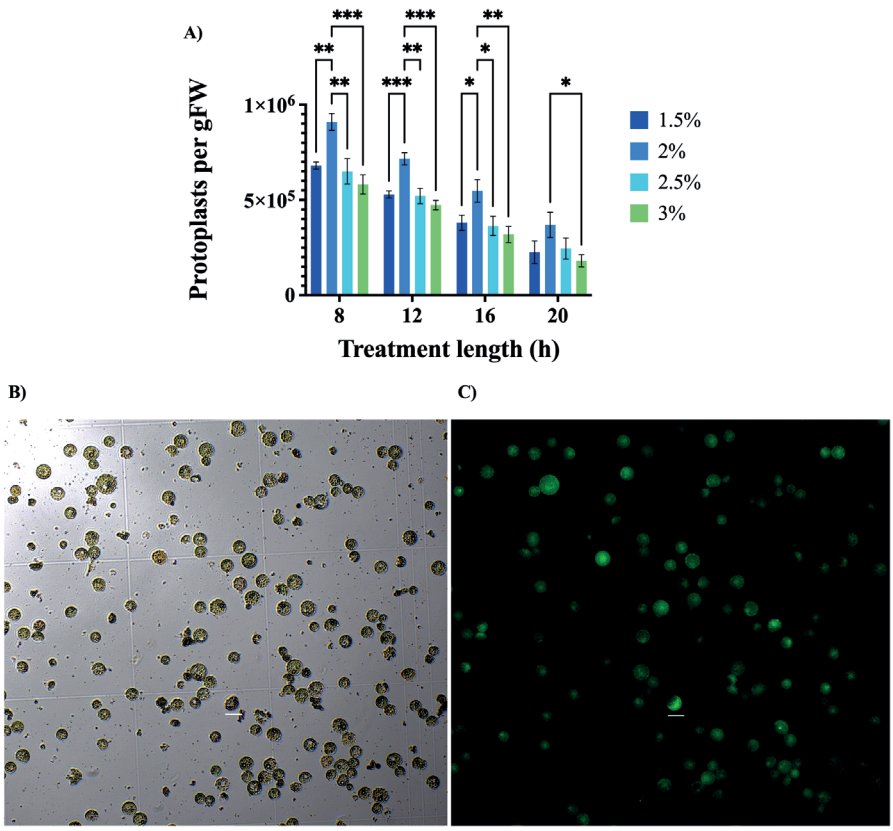

FIGURE 3
Isolation of protoplasts using the classical method. **(A)** Comparison of four enzyme solutions and four different enzymatic treatment lengths. Bars plot portrays results for the different cellulase concentrations used at each time point. The Y-axis shows the number of counted alive cells, using FDA and a hemocytometer, as protoplasts per g of fresh weight (gFW). The X-axis displays the duration of the enzymatic treatment in hours. Error bars represent standard deviation. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001. **(B)** Isolated cells under bright field conditions. **(C)** Viable protoplasts showing fluorescence due to FDA conversion into fluorescein. Scale bar represents 20 µm.

While this result belongs to only one sample, it is a good representation of the possibility of using the piCas9_CRPK1 to simultaneously edit multiple genes. Furthermore, it proves the reliability of our method when analyzing multiplex genome editing reagents.

Importantly, the genome editing efficiency in all cases was enough for proceeding with plant transformation using those vectors. A representation of the indels predicted by TIDE for the two multiplex transformation vectors used in this study can be found in Supplementary file 8.

4 Discussion

Developing reliable genome editing protocols for *Lolium perenne*, an important forage grass, remains challenging due to the limited efficiency of calli transformation. To overcome this, the use of protoplasts has emerged as valuable intermediate step for screening genome editing reagents *in vivo*, such as plasmids and gRNAs. This approach has been successfully used in other grass species like rice, wheat and maize (Shan et al., 2013; Brandt et al., 2020; Fierlej et al., 2022). Here, we describe the development and validation of two approaches for isolating enough perennial ryegrass protoplasts suitable for transformation assays. The PEG-mediated transformation of protoplasts served as a platform to evaluate, *in vivo*, the editing efficiency of three different types of gRNA encoding vectors.

Standard protoplast isolation techniques are based on the enzymatic degradation of cell walls, in combination with gentle mechanical force agitation. In grasses, the most frequent starting material for protoplasts isolation are leaves or tillers. These are normally processed before enzymatic treatment, often by cutting them with a razor blade, to favor enzyme activity. This manual cutting is one of the most labor-intensive and time-consuming parts of most protoplast isolation methods. As a novel alternative for processing tillers of *L. perenne*, we tested the use of a blender to

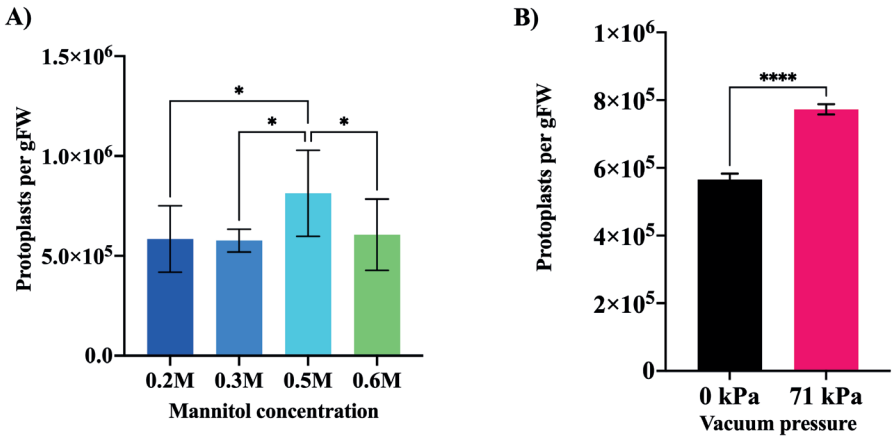

FIGURE 4
Mannitol pretreatment and vacuum infiltration experiments. **(A)** Graph showing the comparison between four mannitol concentrations used for the pretreatment of intact tillers. The Y-axis shows the number of counted alive protoplasts using FDA and a hemocytometer (protoplasts per g of fresh weight, gFW). The X-axis shows the molar concentration of mannitol used per treatment. **(B)** Comparison between the use of 71 kPa of vacuum and the absence of pressure. The Y-axis shows the number of counted alive protoplasts using FDA and a hemocytometer (protoplasts per gFW). The X-axis shows the magnitude of vacuum pressure used in kPa. Each experiment was repeated four times. Error bars represent standard deviation. *P ≤ 0.05 and ****P ≤ 0.0001.

simplify and speed up this process. While this approach decreased the yield of obtained protoplasts by approximately one order of magnitude compared to the razor cutting method, it significantly decreased handling time. The choice between these methods should be based on the downstream applications of the isolated cells. Researchers should consider whether maximizing yield or minimizing labor and processing time is the most important factor for their needs.

The mix of enzymes used for protoplasts isolation varies among different plant species due to differences in cell wall composition (Chawla, 2009; Davey et al., 2010). To optimize this step for *L. perenne*, we tested four different cellulase concentrations: 1.5, 2, 2.5, and 3%. This was supported by previous evidence indicating that cellulose is the main component of *L. perenne* cell walls (approx. 46%) (Gordon et al., 1985), and by informatic models showing an important role for cellulase in degrading perennial ryegrass mesophyll cell walls (Vetharaniam et al., 2014). The rest of the enzymatic solution components were maintained, as previously reported by Yu et al. (2017) and Davis et al. (2020). The enzymatic treatment duration also depends on the plant species, and that is why we tested the effect of different incubation periods. Previously published protocols for *L. perenne* range from 6 h (Yu et al., 2017) to 20 h (Davis et al., 2020) of enzymatic treatment. Based on this variability, we evaluated four different incubation durations: 8, 12, 16 and 20 h. Among the tested combinations, the best performing conditions were 2% cellulase concentration and 8h of enzymatic treatment.

Another important part of the isolation procedure is the use of a pretreatment before the plant material is exposed to the enzyme solution. These pretreatments can be done before or after the tillers are processed or cut and are meant to further improve the number of isolated alive cells since non-ionic solutes have been shown to induce the separation of cell membranes from cell walls (Reed and Bargmann, 2021). We tested a range of mannitol concentrations before processing the tillers or seedlings and found that a 0.5 M solution generated the highest number of alive protoplasts. In addition, we also evaluated the use of vacuum infiltration to enhance enzyme penetration into the plant material. This led to an increase in the protoplast yield. Contrary to previous perennial ryegrass protocols (Yu et al., 2017; Davis et al., 2020), we introduced an additional step consisting of using a sucrose cushion to reduce cellular debris. This helped minimize interference during transformation and downstream use of the protoplasts (Chen et al., 2023).

In line with studies in other plant species, we present a protoplast-based platform for testing the activities of gRNAs prior to their use in non-transient transformations (Brandt et al., 2020; Fierlej et al., 2022; Lee et al., 2023). Although Zhang and colleagues (Zhang et al., 2020) mention the use of perennial ryegrass protoplasts for testing gRNAs, their brief communication does not include data on transformation or editing efficiency. To our knowledge, this is the only study reporting genome editing of *L. perenne* protoplasts. Two other publications presented the transformation of perennial ryegrass protoplasts in a general manner, without focusing on editing purposes (Yu et al., 2017; Davis et al., 2020).

In all these previous studies, PEG was used for the transformation of protoplasts. This method is widely adopted across plant species due to its simplicity and efficiency. Additionally, it does not require specific equipment like electroporation-based transformations. In our study, PEG-mediated transformation was also effective. Fluorescent signal

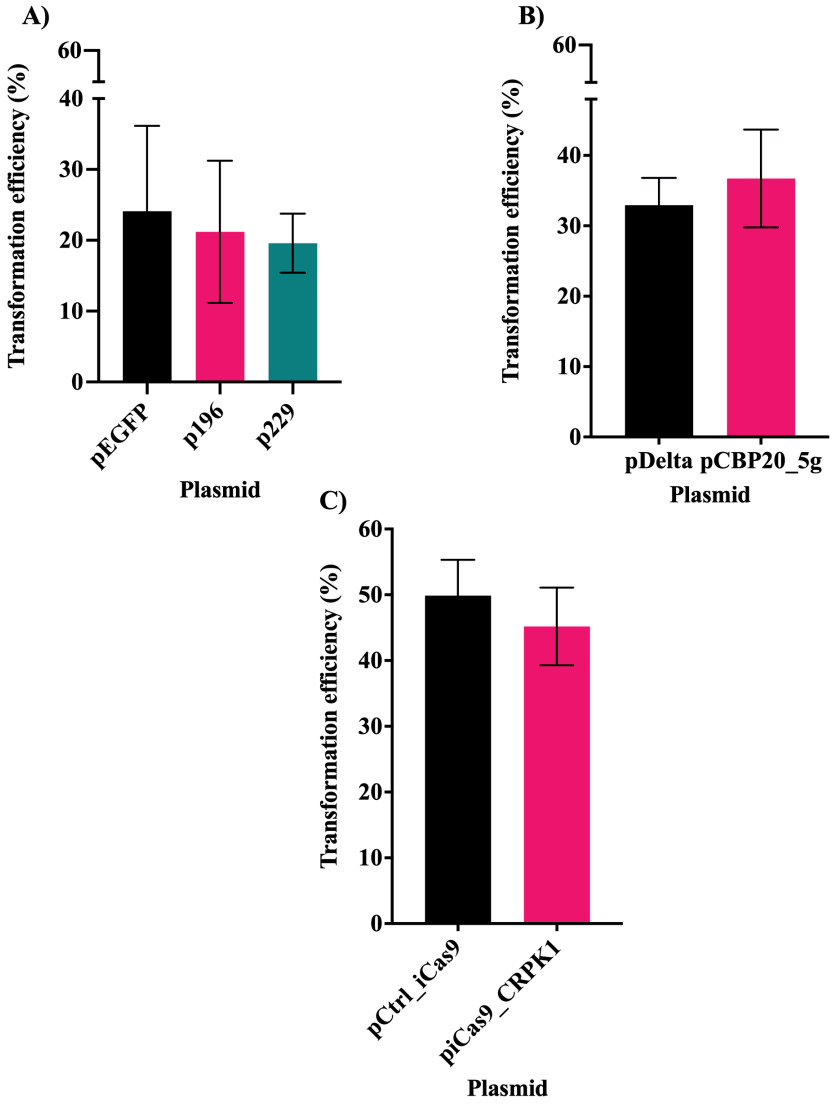

FIGURE 5
Transformation efficiency of the vectors used to transform *L. perenne* protoplasts. **(A)** Transformation efficiency observed when using vectors derived from the pHSE401/EGFP plasmid. pEGFP: control plasmid not coding for any gRNA; p196 coding for gRNA 196 and p229 for gRNA 229. The transformation using the pHSE401/EGFP vectors was repeated 6 times. **(B)** Transformation efficiency observed when using vectors derived from the pTRANS_HiGRFdGm1 plasmid. pDelta: control plasmid not coding for any gRNA; pCBP20_5g coding for five gRNAs. The transformation using the pTRANS_HiGRFdGm1vectors was repeated 12 times. **(C)** Transformation efficiency of the plasmids encoding an intronized Cas9. pCtrl_iCas9: control plasmid not encoding gRNAs; piCas9_CRPK1 coding for six gRNAs. The transformation using the control plasmid was repeated 4 times and 12 times in the case of the vector encoding six gRNAs. The Y-axes show the transformation efficiency derived from the percentage of fluorescent cells observed. Error bars represent standard deviation. No statistically significant differences were observed.

from EGFP or ZsGreen confirmed the successful delivery of the tested reagents.

Fluorescence was detected in over 10% of protoplasts transformed with vectors encoding single gRNAs (p196 and p229), more than 30% of cells transformed with the plasmid encoding 5 gRNAs (pCBP20_5g), and over 40% of protoplasts transformed with the vector targeting *LpCRPK1* (piCas9_CRPK1). These results suggest that the multiplexed vectors may achieve higher transformation efficiency in comparison to those encoding a single gRNA. However, a direct comparison cannot be drawn between the vectors used to edit *LpCBP20*, since they encode two different fluorescent proteins. Nonetheless, there is a possibility that

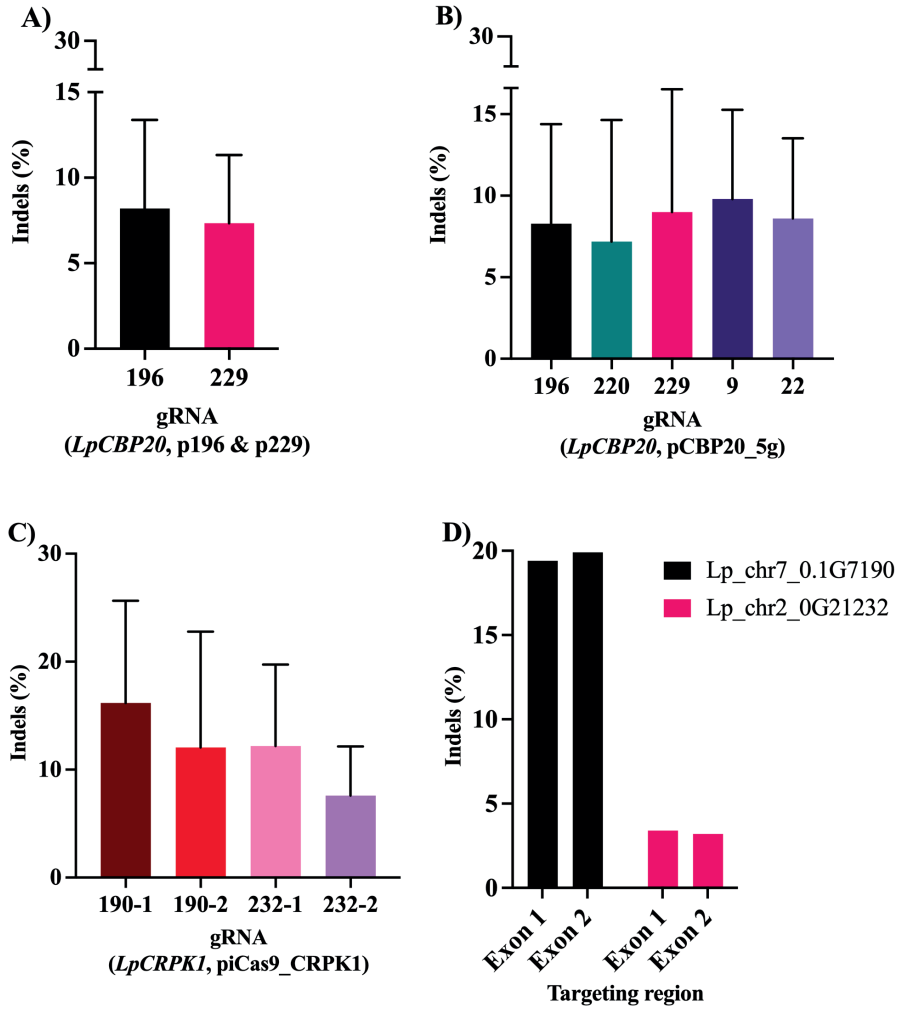

FIGURE 6
Editing efficiency of the different vectors used to transform *L. perenne* protoplasts calculated as frequency of indels with TIDE. **(A)** Editing efficiency of the gRNAs targeting the second exon of gene *LpCBP20* when transforming protoplasts with pHSE401/EGFP-derived plasmids encoding gRNA 196 or gRNA 229. **(B)** Editing efficiency of the five gRNAs targeting *LpCBP20* gene when multiplexed into the pCBP20_5g vector. **(C)** Editing efficiency of gRNAs encoded in the vector piCas9_CRPK1. **(D)** Indels frequencies of the transformation event in which both targeted exons of two of the *LpCRPK1* paralogs (*LpCRPK1–190* and *LpCRPK1-232*) were edited. The data plotted corresponds to results when the cut-offs in TIDE were $P < 0.001$ (for each transformation event) and $R^2 \geq 0.9$. After filtering, the repetitions considered for pHSE401/EGFP-derived plasmids were as follows: n = 5 for 196 and n = 3 for 229. For the pCBP20_5g plasmid repetitions were n = 3 for gRNAs 9 and 22; n = 4 for gRNAs 196 and 229; n = 5 for gRNA 220. For the piCas9_CRPK1 plasmid the repetitions were n = 11 for gRNA 190-1; n = 13 for gRNA 190-2; n = 8 for gRNA 232-1; n = 3 for gRNA 232-2. Error bars represent standard deviation. No statistically significant differences were observed.

the EGFP expression is weaker than usual because of transcriptional silencing. In the pHSE401/EGFP plasmid there are two other transcriptional units under the control of CaMV 35S promoters, in addition to the EGFP cassette (Altpeter et al., 2016; Anjanappa and Gruissem, 2021).This could explain why the vectors encoding a single gRNA presented lower transformation efficiencies than the other types of plasmids.

While no statistically significant differences were detected among the multiplex plasmids, the higher number of fluorescent cells observed when using the intronized nuclease vector may be attributed to improved experimental handling at later stages of the study.

The DNA delivery method used in this study generated enough transformed protoplasts for testing suitable gRNAs. We assessed the

editing efficiency of different guides by Sanger sequencing of genomic DNA followed by TIDE decomposition analysis. This approach provides an estimation of the frequency of indels, induced by a Cas nuclease guided by a specific gRNA, present in a mixed population of cells (Bennett et al., 2020; Brockman et al., 2023; Aoki et al., 2024). TIDE has been reported as particularly well-suited for detecting low-frequency editing events, especially with frequencies below 10%, which was relevant in several of our samples (Brockman et al., 2023; Aoki et al., 2024). In our study, indels were detected in most cases, even though in some samples the results showed low R^2 values, indicating a weaker fit with the statistical model. When comparing the editing efficiency of the vectors targeting the *LpCBP20* gene, no statistically significant differences were observed between the single and multiple gRNA coding vectors. Despite this, the overall performance of both vector types supports their use in downstream applications.

The vector encoding six gRNAs targeting three different paralogs of *LpCRPK1*, achieved editing efficiencies above 10% for three of the gRNAs (190-1, 190–2 and 232-1) and close to 8% in the case of gRNA 232-2. The relatively higher editing rates observed for most of these gRNAs may be attributed to the presence of an intronized Cas9 nuclease in the transformation vector. Previous reports have shown that the addition of introns in the Cas9 coding sequence can enhance gene expression by promoting higher transcription and translation levels, a phenomenon known as intron-mediated enhancement, which can lead to improved editing efficiencies (Rose et al., 2011; Castel et al., 2019; Grützner et al., 2021). The intronized Cas9 used in our study contains thirteen introns and has previously shown editing levels exceeding 90% in barley (Lawrenson et al., 2024). In one of the analyzed samples, two of the targeted paralogs presented indels in both exons, further indicating multiplex editing.

5 Conclusions

From the two different approaches developed and tested for processing perennial ryegrass material before their enzymatic treatment, using a blender was faster and easier but resulted in a lower number of alive cells than the more classical approach based on sectioning the material with a scalpel Additionally, the best composition of an enzymatic solution together with the best performing incubation time were determined. The inclusion of a sucrose gradient, not previously reported in the isolation of perennial ryegrass protoplasts, further increased the isolation of viable cells. Similar to what has been proven in other grasses, the method described in our study showed that perennial ryegrass protoplasts are suitable for testing *in vivo* the outcome of binary vectors used for gene editing. This evaluation allows screening suitable vectors or gRNAs before their application in the transformation of *L. perenne* material, such as calli. The *in vivo* selection of gRNAs can speed up the generation of mutant plants with interesting phenotypes, such as stress-related tolerance, especially in non-model organisms or species recalcitrant to transformation and regeneration. The proposed method can be used to test other editing reagents, such as RNPs, which could allow the generation of transgene-free mutant plants. As the climate is changing faster than plants can adapt to it, having the ability to generate new genotypes with improved stress tolerance is paramount for sustainable food and feed production.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

FS-S: Writing – original draft, Visualization, Formal analysis, Methodology, Writing – review & editing, Conceptualization, Investigation. EE: Investigation, Writing – review & editing, Methodology, Formal analysis, Visualization. LN: Writing – review & editing, Investigation. KK: Writing – review & editing, Investigation. ML: Investigation, Writing – review & editing. MB-S: Writing – review & editing, Investigation. SB: Investigation, Writing – review & editing. DD: Investigation, Writing – review & editing. EK: Investigation, Writing – review & editing. KJ: Funding acquisition, Writing – review & editing. OR: Writing – review & editing, Funding acquisition. NR: Conceptualization, Supervision, Funding acquisition, Writing – review & editing. CS: Conceptualization, Funding acquisition, Writing – review & editing, Formal analysis, Supervision.

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Conflict of interest

The authors declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2025.1744085/full#supplementary-material

SUPPLEMENTARY FILE 1
(a) Graphic representation of the first and second exon of gene *LpCBP20* together with the gRNAs and primers used in this study. (**b**) Graphic representation of the first and second exons of the *LpCRPK1* paralogs targeted in conjunction with the gRNAs and primers used.

SUPPLEMENTARY FILE 2
Sequences of gene *LpCBP20* and *LpCRPK1* paralogs containing location of primers, gRNAs and targeted exons.

SUPPLEMENTARY FILE 3
Map of the pHSE401/EGFP plasmid.

SUPPLEMENTARY FILE 4
(**a**) Map of the transformation vector pCBP20_5g. (**b**) Graphical representation of the transcriptional unit encoding the five gRNAs in the pCBP20_5g plasmid.

SUPPLEMENTARY FILE 5
(**a**) Map of the transformation vector piCas9_CRPK1. (**b**) Graphical representation of the transcriptional unit encoding the six gRNAs present in piCas9_CRPK1.

SUPPLEMENTARY FILE 6
(a) Table containing the primers used in this study. (**b**) Table containing the gRNAs used in this study.

SUPPLEMENTARY FILE 7
Images showing the amplification of the PCR products used for sequencing and subsequent analysis for indel assessment with TIDE.

SUPPLEMENTARY FILE 8
(**a**) Representation of the indel frequencies of one editing event using the pCBP20_5g transformation vector. (**b**) Representation of the indel frequencies of one editing event when targeting paralogs *LpCRPK1–190* and *LpCRPK1–232* using the piCas9_CRPK1 transformation vector.

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Curriculum vitae

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Conference presentations and trainings

2024	Presentations at the EditGrass4Food final conference (Riga, Latvia) and TalTech's annual PhD student conference
2023	Flash-talk at the 35th "EUCARPIA Fodder Crops and Amenity Grasses Section" conference (Brno, Czech Republic)
2022	Poster presentations at the "Mendel Early Career Symposium" (Vienna, Austria) and "New Phytologist Next Generation Scientists" (Tartu, Estonia) PlantEd Crop Transformation / Genome Editing training school, June 13-17, John Innes Centre (Norwich, UK)

Supervision

2023–2025	**Mirjam Nuter**, MSc, "Improving drought tolerance in perennial ryegrass (*Lolium perenne)* through CRISPR-Cas9-mediated genetic modification"
2021–2024	**Erki Eelmets**, MSc, "Isolation and transformation of perennial ryegrass protoplasts for CRISPR/Cas9 applications"
2021–2023	**Olav Kristjan Kasterpalu**, BSc, "Sterilization method of perennial ryegrass tillers for callus induction and downstream applications"

Scholarships

2022	Dora+ Scholarship for training in TEAGASC (Carlow, Ireland) in relation to *Lolium perenne in vitro* culture establishment, handling and transformation. Three weeks.

Publications

Sustek-Sánchez F, Eelmets E, Nigul L, et al (2026) **Isolation and transformation of perennial ryegrass (*Lolium perenne* L.) protoplasts for the *in vivo* assessment of guide RNAs editing efficiency** Front Plant Sci 16. https://doi.org/10.3389/fpls.2025.1744085

Pashapu AR*, Statkevičiūtė G*, **Sustek-Sánchez F**, et al (2024) **Transcriptome profiling reveals insight into the cold response of perennial ryegrass genotypes with contrasting freezing tolerance**. Plant Stress 14:100598. https://doi.org/10.1016/j.stress.2024.100598

Sustek-Sánchez F, Rognli OA, Rostoks N, et al (2023) **Improving abiotic stress tolerance of forage grasses – prospects of using genome editing**. Front Plant Sci 14. https://doi.org/10.3389/fpls.2023.1127532

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Stipendiumid

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Publikatsioonid

Sustek-Sánchez F, Eelmets E, Nigul L, et al (2026) **Isolation and transformation of perennial ryegrass (*Lolium perenne* L.) protoplasts for the *in vivo* assessment of guide RNAs editing efficiency** Front Plant Sci 16. https://doi.org/10.3389/fpls.2025.1744085

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