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Establishing efficient transformation technologies for CRISPR/Cas9 genome editing in *Lolium perenne* L.

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Abstract

The diploid forage grass species perennial ryegrass (*Lolium perenne* L.) is the most important forage grass species in Europe. It is used both in leys and pastures and is a crucial component of ruminant livestock production (milk and meat) due to its good nutritional values and high digestibility. However, its poor adaptation to cold and dry climates poses a challenge in the ongoing climate changes with drier summers and unpredictable winters, such as in Norway. An important aspect for several grasses is the induction of flowering through vernalization, defined as a process where a prolonged cold period promotes flowering in the plant. Freezing tolerance and vernalization have a complex relationship with several molecular cross-talks where many genes are involved and can be induced as a result of exposure to low temperatures as well as shorter days.

In this study, two genes important for vernalization in perennial ryegrass, *VRN1* and *VIN3*, were selected for gene editing using CRISPR/Cas9. Two perennial ryegrass genotypes, Fagerlin-201 and Fagerlin-204, which differ regarding vernalization requirements and expression of these two genes during short and cold days were used as plant material. These genotypes were selected within the Norwegian cultivar 'Fagerlin'. Previous studies have found that the induction of *VRN1* and *VIN3* is significantly higher in the genotype requiring vernalization (Fagerlin-204) compared to the genotype that flowers without vernalization.

For both genes, knock-out and overexpression constructs were designed and used to transform perennial ryegrass calli using *Agrobacterium tumefaciens* mediated transformation. To do so, four gRNAs were designed for each gene. Meristematic calli were produced from seeds from the perennial ryegrass cultivar 'Fagerlin', which were later transformed. Calli from leaves, embryos and meristems were also attempted to be established, however this did not yield the same positive results.

Generating CRISPR/Cas9 constructs, as well as the production and transformation of perennial ryegrass callus, is time-consuming, and demonstrate the challenges of establishing tissue culture methods and CRISPR/Cas9 knock-outs in this species. Therefore, further work should be done on this project such as phenotypic validation of the transformed calli, and

establishment of meristematic calli or protoplast cultures and transformation of the two selected genotypes, Fagerlin-201 and Fagerlin-204.

Establishing a functional CRISPR/Cas knockout system for perennial ryegrass is needed for studying the function of genes characterized through transcriptomic and QTL (Quantitative Trait Loci) studies. Subsequently, such information can be implemented and used to improve the precision of genomic based breeding methods, reduce the length of the breeding cycle, and thus more rapidly develop new cultivars with improved tolerances against stresses like frost and drought.

Sammendrag

Den diploide fôrgressarten flerårig raigras (*Lolium perenne* L.) er den viktigste fôrgressarten i Europa. Den brukes både i eng og beite og er en avgjørende komponent i husdyrproduksjonen fra drøvtyggere (melk og kjøtt) på grunn av god fôrkvalitet og høy fordøyelighet. Den dårlige tilpasningen til kaldt og tørt klima utgjør imidlertid en utfordring i de pågående klimaendringene med tørrere somre og uforutsigbare vintre, slik som i Norge. Et viktig aspekt for flere gressarter er induksjon av blomstring gjennom vernalisering, definert som en prosess hvor en langvarig kuldeperiode fremmer blomstring i planten. Frysetoleranse og vernalisering har et komplekst forhold med flere molekylære samspill der mange gener er involvert og kan induseres som følge av eksponering med lave temperaturer samt kortere dager.

I denne studien ble to gener viktige for vernalisering i flerårig raigras, *VRN1* og *VIN3*, valgt for genredigering ved bruk av CRISPR/Cas9. To flerårige raigrasgenotyper, Fagerlin-201 og Fagerlin-204, som er forskjellige med hensyn til vernaliseringskrav og uttrykk for disse to genene under korte og kalde dager, ble brukt som plantemateriale. Disse genotypene ble selektert innen den norske sorten 'Fagerlin'. Tidligere studier har funnet at induksjonen av *VRN1* og *VIN3* er betydelig høyere i genotypen som krever vernalisering (Fagerlin-204) sammenlignet med genotypen som blomstrer uten vernalisering.

For begge gener ble knock-out- og overekspresjonskonstruksjoner designet og brukt til å transformere kalli av flerårig raigras ved bruk av *Agrobacterium tumefaciens*-mediert transformasjon. For å gjøre det ble fire gRNA-er designet for hvert gen. Meristematiske kalli ble produsert fra frø fra sorten 'Fagerlin', som senere ble transformert. Det ble også forsøkt å produsere kalli fra blader, embryo og meristemer, men dette ga ikke de samme positive resultatene.

Fremstillingen av CRISPR/Cas9-konstruksjoner, samt produksjon og transformasjon av flerårig raigraskallus, er tidkrevende, og demonstrerer utfordringene med å etablere vevskulturmetoder og CRISPR/Cas9-knock-outs i denne arten. Derfor bør det arbeides videre med dette prosjektet, dvs. fenotypisk validering av de transformerte kalliene, og etablering

av meristematiske kalli- eller protoplastkulturer og transformasjon av de to utvalgte genotypene, Fagerlin-201 og Fagerlin-204.

Etablering av et funksjonelt CRISPR/Cas knockout-system for flerårig raigras er nødvendig for å studere funksjonen til gener karakterisert gjennom transkriptom og QTL (Quantitative Trait Loci) studier. Deretter kan slik informasjon implementeres og brukes til å forbedre presisjonen av genomisk-baserte foredlingsmetoder, redusere lengden på foredlingsprogrammet, og dermed raskere utvikle nye sorter med forbedret toleranse mot stress som frost og tørke.

Acknowledgement

I would like to especially thank and express my deep appreciation and gratitude to my main supervisor Mallikarjuna Rao Kovi, as well as my two co-supervisors Manikandan Veerabagu and Odd Arne Rognli for making this thesis possible. For guiding me through this time working in the lab, writing my thesis, motivating me and helping me every step of the way. Their patience and professional guidance throughout my work have been crucial for the completion of this thesis. Through this time, I have gained more knowledge than I could ever expect, thank you for letting me be a part of such a great research environment.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
Bp	Base pair(s)
Cas9	CRISPR-associated protein 9
CBF	C-repeat binding factor
CCT	CO, CO-like and TOC1 domain
cDNA	Complementary DNA
CO	Constans
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
crRNA	CRISPR RNA
dCas9	Dead Cas9
DNA	Deoxyribonucleic acid
DSB	Double-stranded break
FEH	Fructan exohydrolases
FLC	Flowering locus C
FNIII	Fibronectin 3
FT	Flowering locus T
FTs	Fructosyltransferases
gDNA	Genomic DNA
gRNA	Guide RNA
HDR	Homology-dependent repair
Indels	Insertions and deletions
INV	Invertase
Kb	Kilo base pairs
LB	Lysogeny broth
LD	Long day
MADS	Minichromosome-maintenance factor 1 Agamous Deficiens Serum response factor
mRNA	Messenger RNA
MS	Murashige and Skoog
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
OD	Optical density
PAM	Protospacer-adjacent motif
PCR	Polymerase chain reaction
PCR2	Polycomb-group repressive complex 2
PHD	Plant homeodomain
RFP	Red fluorescent protein
RNA	Ribonucleic acid

RNP	Ribonucleoprotein
SAM	Shoot apical meristem
SD	Short day
SOC1	Suppressor of overexpression of constans1
SSN	Sequence-specific nuclease
TALENS	Transcription activator-like effector nucleases
t-DNA	Transfer DNA
tracrRNA	Trans activating crRNA
tRNA	Transfer RNA
VIN	Vernalization insensitive
VRN	Vernalization response
ZFN	Zinc finger nuclease

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1. Introduction

1.1 Importance of perennial ryegrass

Lolium perenne L. (perennial ryegrass), is a diploid (2n=2x=14) grass species with a self-incompatibility mating system preventing self-fertilization and ensuring genetic variation (Guthridge et al., 2001). It is one of the several important forage grasses for ruminant production in Norway, as well as the most important forage grass in Europe (Humphreys et al., 2010). It is also becoming increasingly important in the Nordic-Baltic regions (Helgadóttir et al., 2018), and also in New Zealand and the south-eastern part of Australia (Guthridge et al., 2001), for its high digestibility and nutritional values. It is part of the basis for 70 % of meat production and 80 % of milk production worldwide (Zhang et al., 2020). Grasses are also of importance in nature due to their generation of oxygen, and carbon storage, making grasslands important biomes and CO_2 sinks (Sustek-Sánchez et al., 2023).

Norwegian agriculture is dominated by grass-based livestock and milk production. Perennial ryegrass is an important forage crop due to its high forage quality (Kovi et al., 2015), with good digestibility and positive nutritional values. However, the species exhibits poor adaptation to unfavourable environmental conditions such as dryer and colder climates. Growing perennial ryegrass can also become challenging considering ongoing climate changes, such as drier summers and more unpredictable winters, especially in Northern Europe. Large economic consequences might occur if perennial ryegrass varieties don't adapt to the fluctuating unpredictable winter temperatures (Thorsen and Höglind, 2010). Low temperatures are a limitation for the productivity of forage crops and have several consequences such as tissue damage, metabolic imbalances, reduced germination, and delay or prevention of reproductive development (Andrews, 1987).

1.2 Vernalization and photoperiod responses determine flowering time

Vernalization can be defined as a process where a growing plant or hydrated seed use prolonged cold periods to promote flowering. This contributes to determining both growth habits and flowering time in grasses (Barrett et al., 2002). The ability to cold acclimate makes plants able to survive freezing with temperatures below zero. Vernalization is the promotion of flowering at low temperatures, which is characterized as an epigenetic response. The

length of the vernalization requirement determines how quickly the plants can flower the following spring. Annual grasses like the *Lolium multiflorum* cultivar “Westerwold” do not have a vernalization requirement, as they flower in the same year and do not survive the following winter (Landman et al., 1995). The relationship between the vernalization response and freezing tolerance is complex (Barrett et al., 2002). Several molecular and physiological responses are initiated by above-freezing cold temperatures which triggers rapid induction of several genes (Kim and Sung, 2014).

If plants that require vernalization are not treated with low temperatures, flowering will remain repressed and the plants will stay vegetative, or display delayed flowering (Taiz et al., 2018). Most varieties of perennial ryegrass require vernalization and flowers in the spring after a cold period (Heide, 1994, Aamlid et al., 2000). Flowering is an important aspect of perennial ryegrass since it affects seed yield and the net energy for metabolism and growth (Herridge et al., 2021).

Photoperiodism is the ability of plants and other organisms to detect the length of the day which enables seasonal response since it makes it possible for certain events to occur at specific times of the year. In the 1920´s it was discovered by Garner and Allard (Taiz et al., 2018) that day length is what determines flowering, rather than photosynthate accumulation. One can differentiate between two types of flowering plants: short-day plants (SD) and long-day plants (LD). SDs will only flower or have accelerated flowering in short days , whereas LDs require day lengths that exceed the critical day length (Taiz et al., 2018). It has been discovered that day length is measured by the plants themselves; they measure the length of the night rather than the length of the day as the names imply.

An important gene involved in flowering in SDs and LDs is the *FLOWERING LOCUS T* (*FT*), which is the main floral signal encoding the stimulation of flower evocation in the meristem, known as the florigen (Tsuji, 2017). The florigen is transported to the shoot apical meristem (SAM), after being synthesized in the plant leaves. In the SAM the plant will transition to reproductive growth from the vegetative state (Tsuji, 2017).

FT is promoted by the transcriptional regulator *CONSTANS* (*CO*) which promotes flowering in *Arabidopsis* and regulates the transcription of other genes (Taiz et al., 2018). Homologous to this in SDs such as rice (*Oryza sativa*) are the genes *Heading-date1* (*Hd1)* and *Heading-date3a* (*Hd3a*). An overexpression of *FT/Hd3a* will result in prompt flowering irrespective of photoperiod, meaning that these genes are strong flowering promoters (Taiz et al., 2018).

Another important set of genes is the *FLOWERING LOCUS C* (*FLC*) genes, found in *Arabidopsis thaliana*. The *FLC* genes are important regulators in many aspects of plant development, the most known being with vernalization-regulated flowering (Kennedy and Geuten, 2020). *FLC* genes, described as a clade of MADS-box transcription factors (Kennedy and Geuten, 2020), act by repressing the activation of important genes for floral promotion such as the *FT* genes and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). It was first believed that the presence of *FLC* was restricted to eudicots such as *Arabidopsis*, however, phylogenetic relatives of *FLC* exist in cereals and other monocots, divided into the two subclades OsMADS51 and OsMADS37 (Kennedy and Geuten, 2020).

Flowering mechanisms in other model plants and grasses, such as *Brachypodium distachyon* and *Arabidopsis thaliana*, are well studied (Herridge et al., 2021). This has led to the discovery of several important genes involved in to vernalization and flowering responses. Important flowering genes have also been found in related crops like rice (*Oryza sativa*), wheat (*Triticum* sp.), barley (*Hordeum vulgare*) and perennial ryegrass (*Lolium perenne*). The four important vernalization genes affecting flowering time are *VRN1, VRN2, VRN3* and *VIN3* in both cereals and grasses.

The vernalization response (*VRN1*) loci affects flowering and maturity. The gene is located in the middle of the long arms of the wheat chromosomes 5AL, 5BL, and 5DL (Dubcovsky et al., 1998, Galiba et al., 2009, Islam, 2015). Allelic differences on *VRN1* is the reason why some plants have a vernalization requirement and some do not (Galiba et al., 2009). The *VRN* genes can be found in diploid wheat (*Triticum monoccum*) as well as in barley (*Hordeum vulgare*) (Yan et al., 2003, Trevaskis, 2010). A phylogenetic tree with related gene

orthologues to the *VRN1* gene in perennial ryegrass and other grass species shows that they have a similar gene related to flowering (Figure 1).

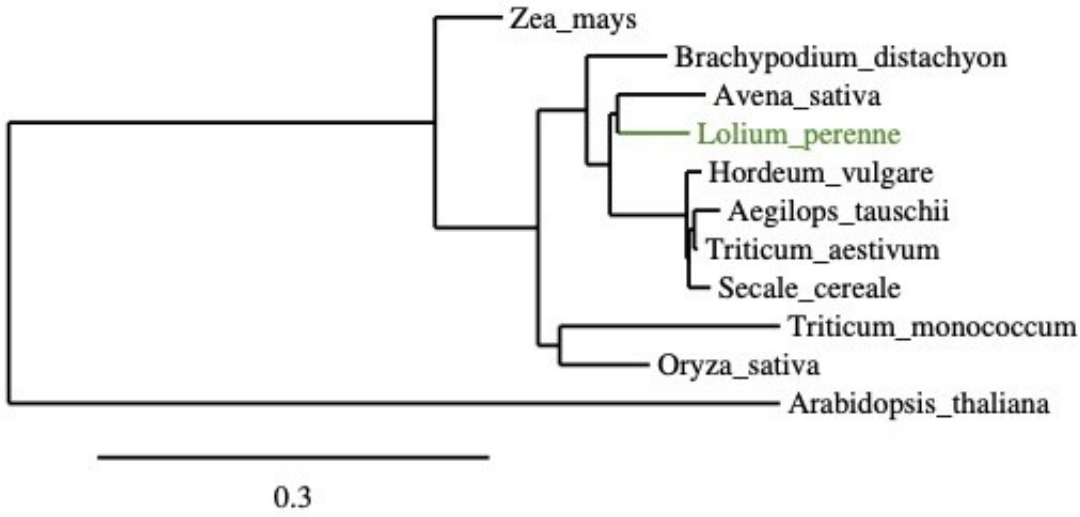

Figure 1. Phylogenetic tree showing orthologues to the Lolium perenne VRN1 gene in other grass species. Sequences were retrieved by using the BLAST tool from NCBI (Altschul et al., 1990).

VRN1 is a MADS-box transcription factor that is induced by low temperatures (Herridge et al., 2021). When exposed to longer periods of colder temperatures the expression of this gene will be stable. *VRN1* alone, as a floral promoter (Kennedy and Geuten, 2020), is responsible for the time of maturation and flowering, and the expression of the gene is regulated by vernalization and development. Further, when transitioned into longer days the *VRN1* gene is upregulated and can activate the *FLOWERING LOCUS T* (*FT*) in leaves, as seen in **Error! Reference source not found.** Herridge et al. (2021). In perennial ryegrass, the orthologue *VRN3* gene is homologous to the *FT* gene in *Arabidopsis* (Islam, 2015). This gene acts as a transcriptional co-activator by being transported from the leaves where it is activated, to the apical meristem.

Figure 2 shows how the expression of *VRN1* differs in the 2 different genotypes of the perennial ryegrass cultivar 'Fagerlin'. In Fagerlin-204, which requires vernalization, the expression of *VRN1* is significantly higher during short days and low temperatures (mid-December until mid-January). Genotype Fagerlin-201, which can flower without

vernalization, has a much lower induction of the *VRN1* gene in this period compared to Fagerlin-204 (Islam, 2015).

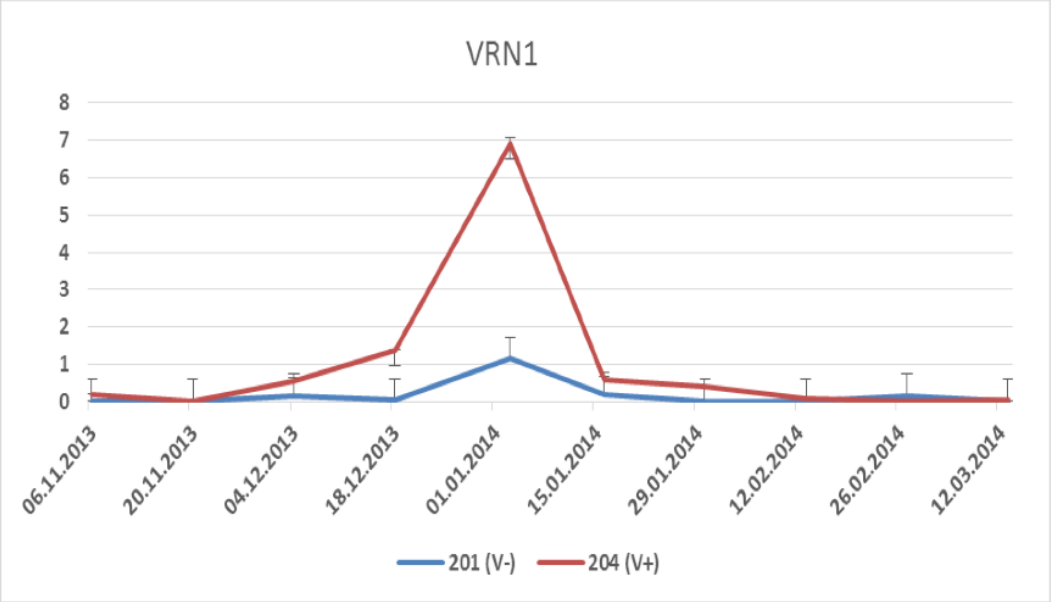

Figure 2. Expression patterns of VRN1 in two perennial ryegrass genotypes of cv. Fagerlin, that differ in vernalization requirements. Genotype Fagerlin-201 flower without vernalization (V-), while genotype Fagerlin-204 require vernalization to flower (V+). The Y-axis represents the fold change in the expression of each genotype, while the X-axis shows sample collection time points. This figure was retrieved from Islam (2015).

Another vernalization gene, *VRN2*, has an important role in blocking the flowering in long days, doing so by repressing the *VRN3*/*FT* gene, which creates a requirement for vernalization. *VRN2* can be downregulated by low temperatures and short days through *VRN1* gene activity, meaning that *VRN1* can repress the expression of *VRN2* (Barrett et al., 2002, Herridge et al., 2021), further leading to the *VRN2*s repressive effect on *VRN3* (Kennedy and Geuten, 2020). *VRN2* is characterized as a zinc-finger CCT-domain protein (Herridge et al., 2021). The CCT domain is found in several genes in other plants and variations here can have different impacts on vernalization responses in these plants. The *VRN1* gene was identified in perennial ryegrass and the vernalization response shown to be affected in this species by natural variations in the first intron (Jensen et al., 2005).

Another important gene related to vernalization and the *FLC* gene is the *VERNALIZATION INSENSITIVE 3*, *VIN3*, gene. *VIN3* is a chromatin remodelling protein induced by low temperatures, that is required in *Arabidopsis* for its response to vernalization (Bond et al.,

2009). *VIN3* has a plant homeodomain (PHD) and fibronectin 3 (FNIII) domains and is part of a family of plant-specific proteins (Wood et al., 2006). FNIIIs are often involved in interactions of the protein-protein type, while PHDs are usually found in complexes of chromatin remodelling (Sung and Amasino, 2004). In the vernalization-mediated flowering pathway in *Arabidopsis*, it is the far most up-stream gene (Islam, 2015), and it binds itself to the chromatin and the first intron in the *FLC* locus (Wood et al., 2006, Bond et al., 2009). Figure 3 shows a phylogenetic tree illustrating the proximity of similar genes to *VIN3* in other species.

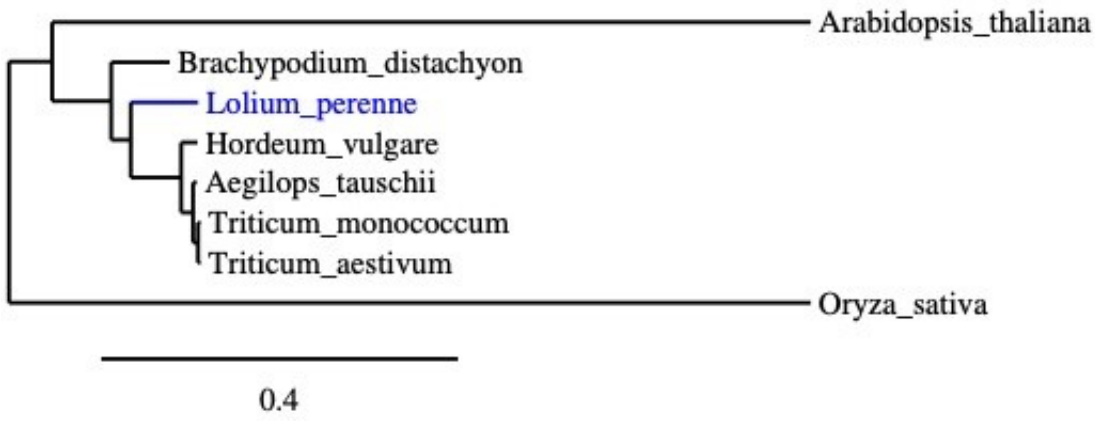

Figure 3. Phylogenetic tree showing orthologues to VIN3 from Lolium perenne in similar species. Sequences were retrieved by using the BLAST tool from NCBI (Altschul et al., 1990).

VIN3 will only be expressed after exposure to cold temperatures that influence vernalization and therefore works as a marker (Sung and Amasino, 2004). This means that *VIN3* has an important role when it comes to separating short fluctuations in temperature, such as in the autumn, from longer colder periods in the winter, and repression of *FLC* will therefore not occur until after induction of *VIN3* (Sung and Amasino, 2004). In this way, *VIN3* has a role in epigenetic gene regulation since it ensures proper timing for flowering by maintaining an epigenetic memory of the prolonged cold exposure.

During vernalization treatment, the amount of *VIN3* mRNA increases but decreases again when temperatures return to normal, which may be part of a mechanism that inhibits plants from flowering under short temperature fluctuations (Wood et al., 2006). When binding to the *FLC* locus chromatin it interacts with parts of the conserved PCR2, which stands for polycomb-group repressive complex 2 (Bond et al., 2009). This complex then trimethylathes a repressive chromatin mark, histone H3 lysine 27 (H3k27me3) (Bond et al., 2009), which will

increase at the *FLC* locus because of vernalization. Later, when plants are exposed to warmer temperatures *VIN3* will quickly be repressed and during growth in warm temperatures, the abundance of *VIN3* mRNA is very low (Bond et al., 2009, Sung and Amasino, 2004, Wood et al., 2006).

Figure 4 shows the gene pathway of the vernalization genes and their interaction, both when exposed to long and short days.

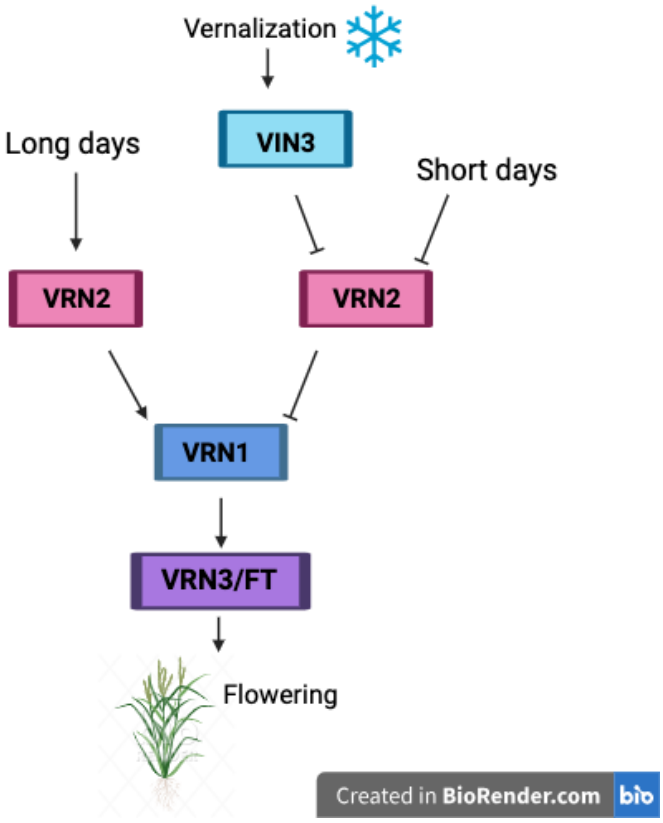

Figure 4. Gene pathway of vernalization genes. VIN3 will be expressed after a prolonged period of cold temperatures and short days, which further leads to the vernalization response by delaying flowering. This is done by repressing VRN2, which in turn represses VRN1. This delays the flowering since VRN3 will not be expressed. This illustration was created with Biorender.

Figure 5 shows how the expression of *VIN3* differs in the two "Fagerlin" genotypes. During the cold period from which the samples were retrieved, the expression of *VIN3* is much higher in the genotype requiring vernalization, 204, compared to 201, where the vernalization requirement is absent (Islam, 2015).

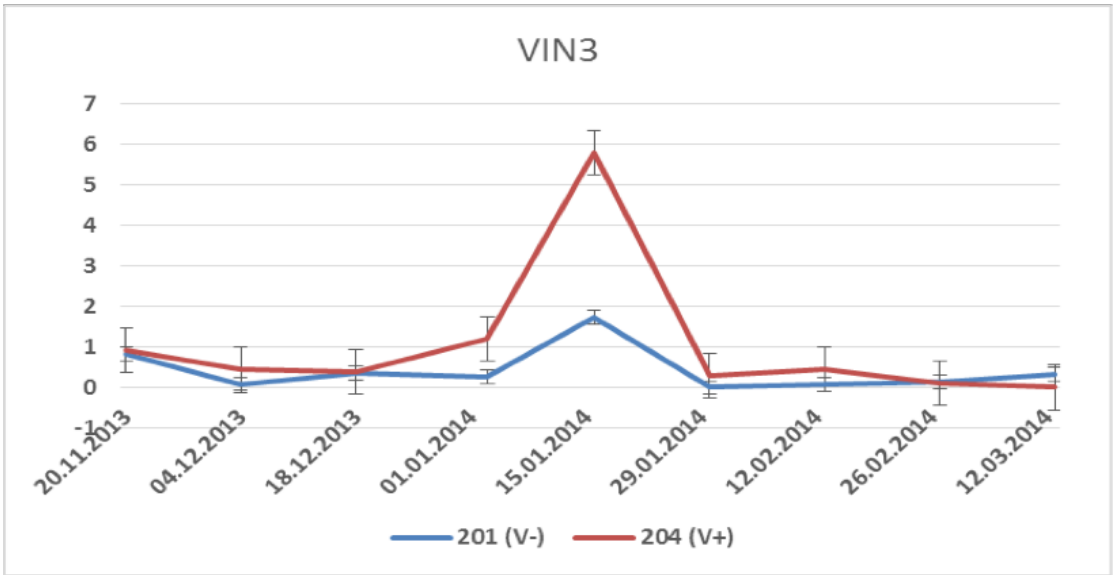

Figure 5. Expression pattern of VIN3 in perennial ryegrass in two genotypes of the "Fagerlin" cultivar, that differ in vernalization. The Fagerlin-201 genotype flower without vernalization (V-) and the Fagerlin-204 genotypes require vernalization to flower (V+). The Y-axis represents the fold change in the expression of each genotype, while the X-axis shows sample collection time points. This figure was retrieved from Islam (2015).

These genes are also related to the *CBF* regulon consisting of the genes *CBF1, CBF2,* and *CBF3* amongst others, which contribute to acclimation to cold temperatures (Park et al., 2018). These genes were first studied in *Arabidopsis,* and they 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). There are many *CBF* genes in temperate cereals, and some genes are only found in the grass subfamily Pooideae, including perennial ryegrass (Galiba et al., 2009).

Fructans, found in temperate forage grasses such as *Lolium*, are synthesized from sucrose and can be defined as non-structural storage carbohydrates. They are stored in the vacuole in plant cells and will either have linear or branched fructose polymers with glycosidic bonds to sucrose (Islam, 2015). Fructans can help plants adjust to stress conditions such as cold stress and drought and are usually accumulated in the roots and leaves of grass species (Islam, 2015). Accumulation of fructans involves fructosyltransferases (FT*s*), invertases (INV), and fructan exohydrolases (FEH) genes. These are all regulated tightly and the genes regulating them have been characterized and isolated (Chalmers et al., 2005).

1.3 Current challenges in perennial ryegrass adaptation

1.3.1 Climate change

Hansen-Bauer et al. (2017) contributed to a report made for the Norwegian Miljødirektoratet about the changes in Norwegian climate for the next century, by using historical data, statistical analyses, and emission scenarios to predict future climates changes in Norway. Although there are some uncertainties regarding this report's predictions of the future, related to natural variations, climate models as well as future emissions, it gives an overview of expected changes in different aspects of the Norwegian climate. Figure 6, shows how annual temperatures have differed from the mean temperature from 1900 to 2010.

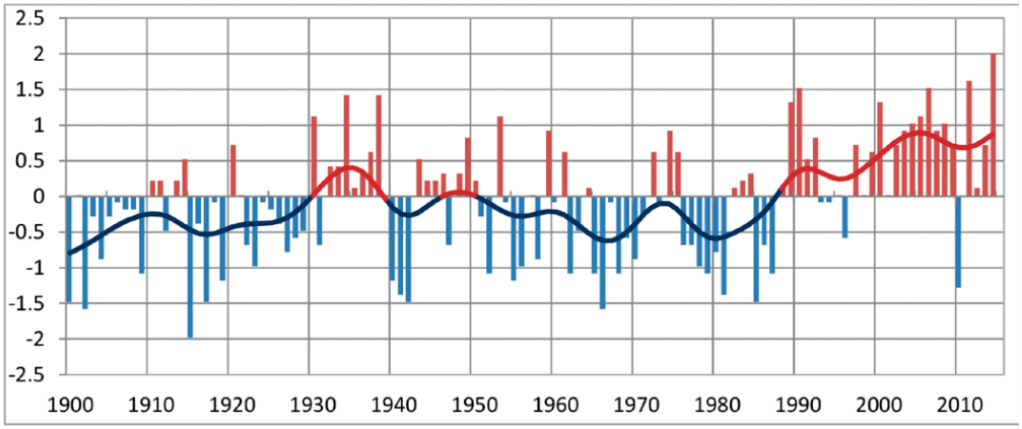

Figure 6. Annual temperatures in Norway from 1900-2014, show deviations from the mean temperature. Figure from Hansen-Bauer et al. (2017).

Using two different emission scenarios, RCP4.5 and RCP8.5, it is predicted that the annual temperatures in Norway will increase respectively by 2.7 °C and 4.5 °C until the end of the century, as shown in Figure 7. Further, it is stated that the general projections for all seasons indicate a warmer climate in Norway and that the winters will experience a greater sense of warming than the summers, meaning that the differences will be greater in the colder seasons (Hansen-Bauer et al., 2017). It is also predicted that the number of warm days per year, meaning days where the daily temperature exceeds 20 °C, will increase until the year 2100.

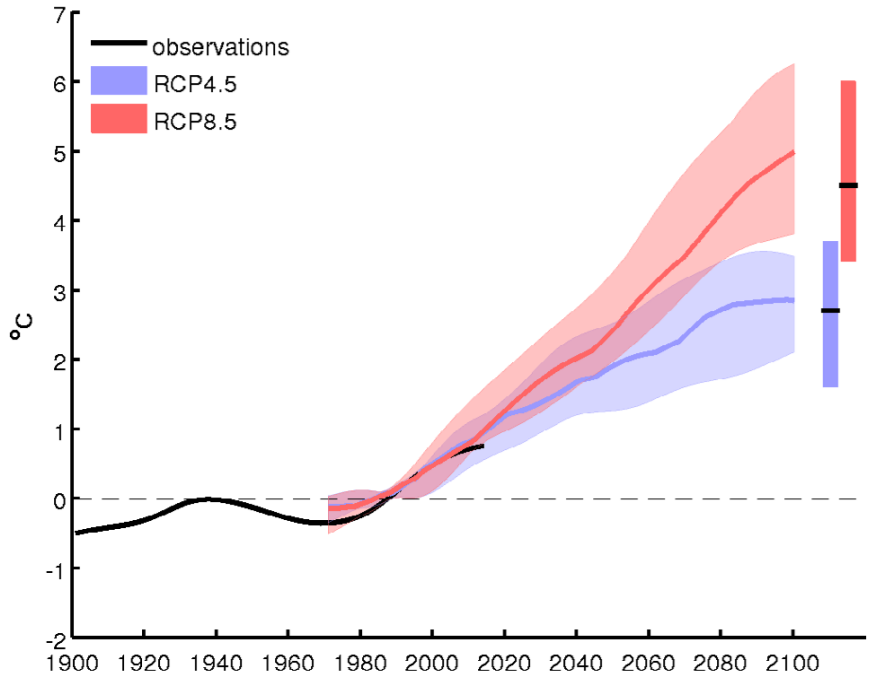

Figure 7. Figure from Hansen-Bauer et al. (2017) showing deviations from the mean temperature (0 °C) in Norway, using the two emission scenarios RCP4.5 and RCP8.5, until the year 2100.

It is also shown that growing seasons will become months longer with some differences in how many months regarding which emission scenario is used (Figure 8) (Hansen-Bauer et al., 2017). Another climate factor that is predicted to increase is the amount of precipitation, with an increase for all four seasons, as well as the amount and intensity of heavy rainfall.

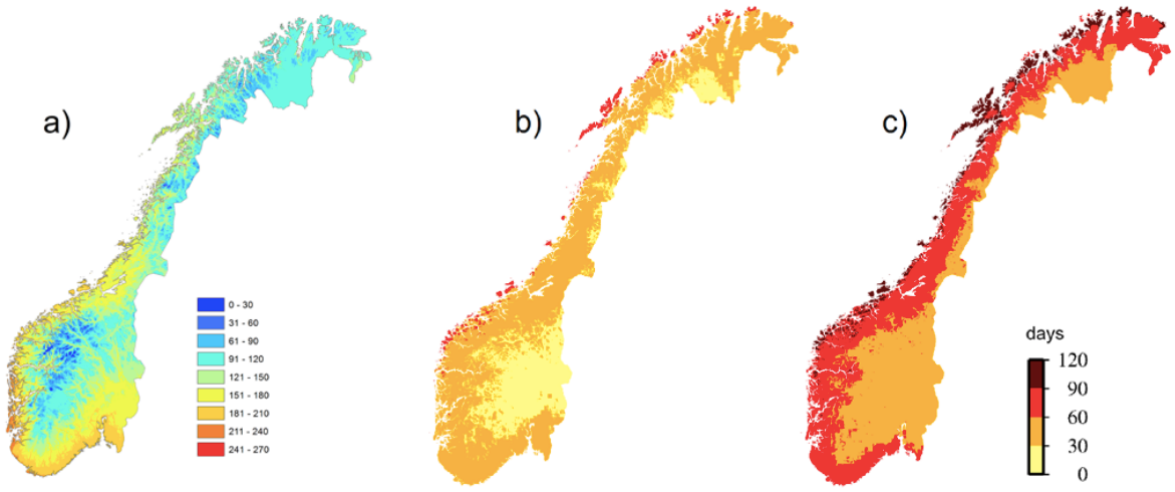

Figure 8. *Figure from* Hansen-Bauer et al. (2017) *showing reference length of the growing season in Norway (a), increased growing season using scenario RCP4.5 (b), and RCP.8.5 (c). From the figure one can see that the biggest changes are between a) and c) where RCP.8.5 is illustrated. However, both scenarios illustrate that the growing seasons in Norway will increase with at least <30 days throughout the country.*

Drought is defined as a deficit of water over a large area for a prolonged time (Hansen-Bauer et al., 2017). It is explained that despite more precipitation, soil moisture will not automatically increase due to the higher temperatures. The emission models, especially RCP8.5, indicate larger deficits of soil moisture near the end of the century, which increases the need for irrigation to avoid drought.

As shown from this report there are several challenges to overcome when developing new perennial ryegrass varieties, especially regarding the time frame of traditional breeding to meet the demands of a rapidly changing Norwegian climate (Rognli et al., 2018). These changes can all affect the productivity of perennial ryegrass, with drought giving a reduced yield and milder winters removing the important cold temperatures needed for vernalization and cold acclimation, which might lead to plants requiring vernalization not being able to flower and produce seeds. Another important factor is if plants are exposed to direct frost or warm spells creating ice covers (Robotham et al., 1978). To accelerate the production of new perennial ryegrass varieties that can more easily adapt to these sorts of predictions for the future climate, more advanced breeding and molecular technologies are to be implemented.

1.3.2 Breeding

Breeding programs on perennial ryegrass have been executed since the 1920s (Zhang et al., 2020), and have provided improved, adapted cultivars with higher yield potentials, persistence and better resistance to several types of biotic and abiotic stresses, such as rust resistance, different heading times, and other characteristics. However, there are several challenges related to developing more suitable varieties, especially rapidly enough considering the ongoing climate changes, as well as lowering the costs of producing new varieties.

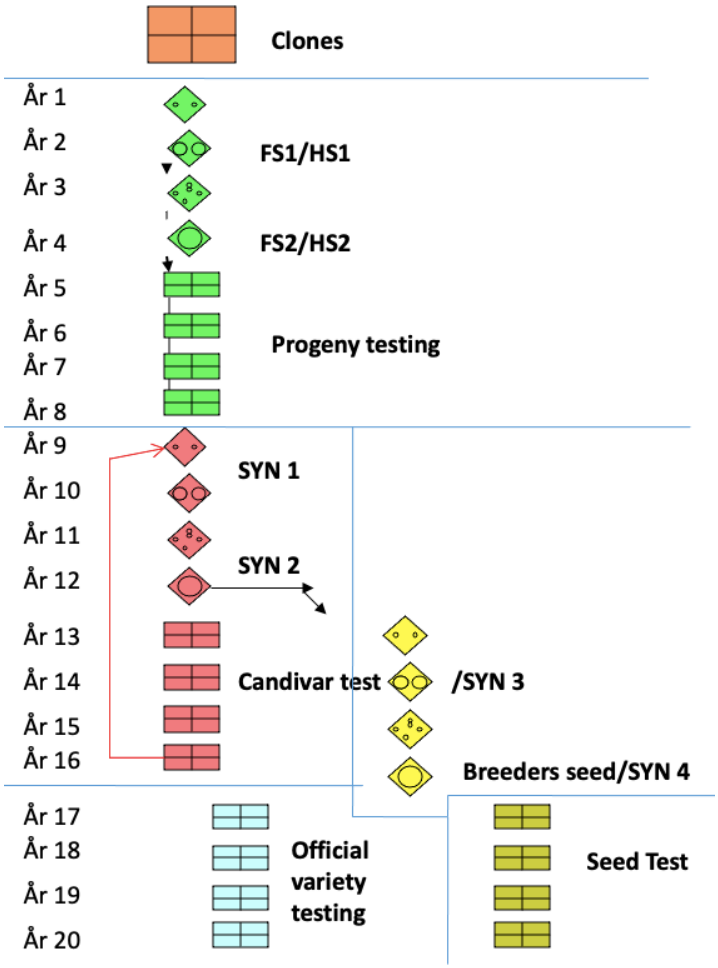

Figure 9. Illustration of the period required for a breeding program for perennial ryegrass. Four years to generate full sib/half sib 2 (FS2/HS2) families and another 4 years for progeny testing. Further, 4 years for generating synthetic population 2 (SYN2) and 4 years for pre-cultivar testing. As seen, in total nearly 20 years is necessary from crossing selected clones until official variety testing as well as the seed testing. Credit to Odd Arne Rognli and Petter Marum.

One of the reasons breeding programs in forage grasses are time-consuming, laborious, and expensive is the outcrossing mating system, governed by self-incompatibility genes found in most forage grass species (Zhang et al., 2020). This mechanism helps to reject fertilization by pollen from a plant landing on the stigma of itself and is important for preventing inbreeding (Franklin-Tong and Franklin, 2003). The most important reason for the long time span of breeding cycles is the need for extensive field testing. Figure 9 shows an illustration of the time required for a complete breeding program in perennial ryegrass. Compared to cereals, where field trials of the varieties are tested in 2 or 3 years, a similar testing of grass cultivars will take 6 to 9 years. This is caused by the cutting of the plants that occur 3-4 times a year during a testing period of 3 years.

Yet another factor is the vernalization requirement, meaning that 2 years are required in order to make crossings compared to 1 year in cereals. Traditional forage grass breeding takes nearly 20 years. Figure 10 shows an illustration of breeding using gene editing, where CRISPR/Cas9 can shorten the breeding process with 4-6 years (Sustek-Sánchez et al., 2023).

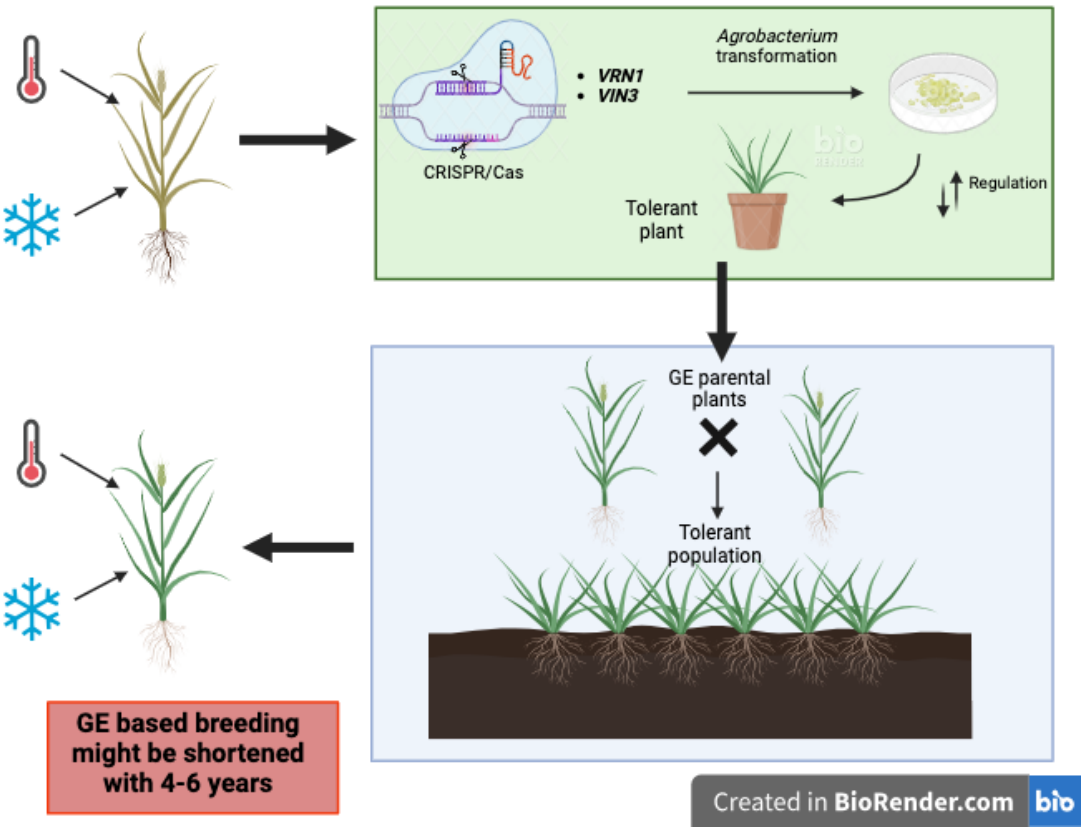

Figure 10. Illustration showing that gene editing methods such as CRISPR/Cas9 is a less time-consuming process, that could shorthen the breeding cycle with approximately 4-5 years, if reproductive traits are targeted. Biotic stresses such as frost and low temperatures prevent the well-being of non-tolerant grass. Different genes can be targeted using the CRISPR/Cas9 system. Agrobacterium-mediated transformation is a suitable delivery method of the CRISPR/Cas9 gRNAs complex for alterations leading to a tolerant plant. Once such tolerant parental plants are obtained, they can be crossed to produce a population able to overcome the effects of abiotic stresses. This illustration is retrieved and a modified version of the figure from Sustek-Sánchez et al. (2023). Illustration made with Biorender.

1.4 CRISPR/Cas9 based genome editing

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a relatively new genome editing method developed in 2012 by Jennifer Doudna and Emmanuelle Carpentier (Doudna and Charpentier, 2014). CRISPR/Cas9 is a gene editing technology that makes it possible to turn genes on or off in cells relatively quickly and easy (Redman et al., 2016). Figure 11 gives an overview of the CRISPR/Cas9 method and how a double-stranded break

(DBS) can be repaired both with non-homologous end joining (NHEJ) and homologous dependent repair (HDR).

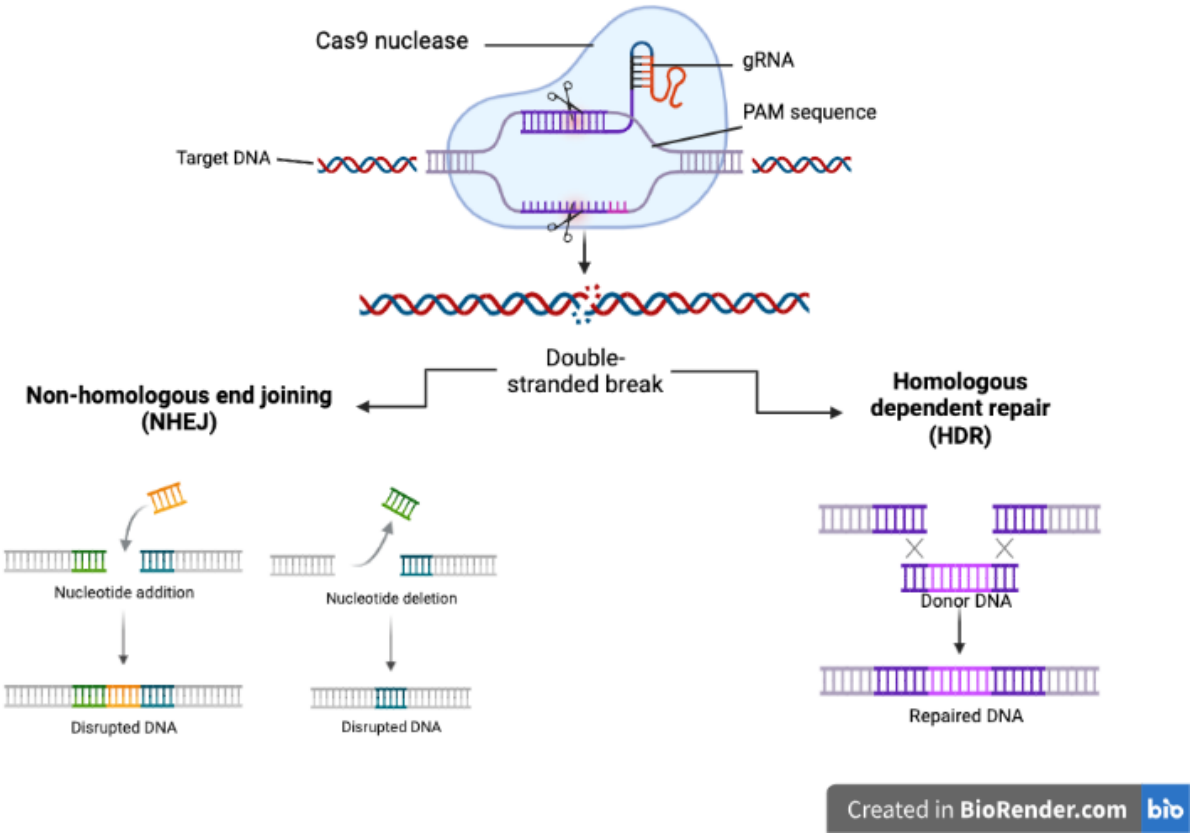

Figure 11. Schematic illustration of the repair mechanisms of double-stranded breaks (DBS) in DNA. The Cas9 nuclease is directed to the target DNA region by base pairing. The Cas9/sgRNA recognizes the PAM sequence downstream of the gRNA and creates a (DSB). This DSB is subsequently repaired by either the error-prone Non-Homologous End Joining (NHEJ) or the Homology-Dependent Repair (HDR) pathways. (Created in Biorender).

The first CRISPRs were discovered in *Escherichia coli* over 30 years ago when analyzing genes related to the isozyme conversion of alkaline phosphatase (Ishino et al., 2018). It was discovered that in different clones of the genes, the same sequence would appear many times, spread out with unique sequences in between. However, due to the lack of advanced sequencing and DNA analysis tools, the biological significance of the sequences remained unknown for several years (Ishino et al., 2018).

CRISPR/Cas9 is described as a DNA-based archaeal and bacterial adaptive immune system that defends against mobile genetic invaders, such as plasmids and bacteriophages (Dort et al., 2020). A big advantage of the CRISPR/Cas9 system is its ability to simultaneously target

multiple sites (Čermák et al., 2017). CRISPR covers several different methods of genome editing allowing double-stranded and precise cuts in an organism's genome (Spencer, 2020), one of them being the knock-out method that inactivates a gene completely. To edit a genome using CRISPR/Cas9 a gRNA, Cas-protein, and a PAM-sequence are needed.

Many approaches to genome engineering use sequence-specific nucleases (SSNs) to create targeted DSBs in genomes. Both transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 reagents are SSNs (Čermák et al., 2017, Langner et al., 2018). The Cas protein makes targeted DSBs in genomes and the desired sequence modification is then achieved by DNA repair (Čermák et al., 2017). There are two ways of repairing DSBs; homologous dependent repair (HDR) and non-homologous end joining (NHEJ). HDR uses sequence information from a donor or sister chromosome to fix the DSB. NHEJ can create indel mutations at the site of the DBS and can knock out the gene function. NHEJ happens at higher frequencies than HDR plant cells, meaning that HDR is harder to achieve since both the DNA molecule and the SSN expression cassette must be introduced into the plant cell (Čermák et al., 2017). Therefore, when knocking out a gene using CRISPR/Cas, NHEJ is utilized to create indels disturbing the reading frame, causing a frameshift, and creating a loss of function for the gene.

The CRISPR/Cas9 method is useful since the target specificity is not determined by proteins, but rather by short guideRNAs (gRNAs) (Čermák et al., 2017). For each new target, a protein must be engineered, however, with gRNAs this is not needed. The gRNA recognizes the target sequence, which then makes it possible for the CRISPR-associated (Cas) endonuclease to cut the sequence at this location. For the Cas-protein to be able to cut at the site, which is recognized by the gRNA, the PAM-sequence is required. The most widely used and well-characterized Cas-protein is the *Streptococcus pyogenes* Cas9 (SpCas9) (Miller et al., 2020), and for this protein, the PAM-sequence is 5'-NGG-3', where N is any base. The Cas9 protein forms a ribonucleoprotein complex with the gRNA and will then be able to perform a double-stranded break adjacent to the PAM sequence (McCarty et al., 2020).

The gRNA sequences consists of a scaffold sequence as well as an adaptor sequence. The scaffold sequence is a non-variable part derived from the trans-activating crisprRNA

(tracrRNA) which binds the gRNA to the Cas9 protein. The tracrRNA was first discovered in the *Streptococcus pyogenes* bacteria in 2011 (Liao and Beisel, 2021). The crisprRNA (crRNA) makes the adaptor sequence of ~20 variable nucleotides which binds the target DNA and CRISPR/Cas9 complex together.

When targeting a gene using CRISPR/Cas9 one can either use single gRNAs or multiple gRNAs targeting the same gene, where numerous gRNAs will be expressed at once, which is called multiplexing (McCarty et al., 2020). Since many plants are polyploid (Dar and Rehman, 2017) and have several copies of the same genes, knocking out a gene and its function can be challenging. When doing CRISPR/Cas9 in plants, multiplexing could be a solution to ensure knocking out all homologs with multiple gRNAs targeting the same gene. However, there are also some disadvantages such as increasing the number of off-targets and risking changes in other parts of the genome.

When designing gRNAs, several practices can increase the chance of generating knock-outs. If gRNAs are located close to the start codon in the coding sequence of the gene, the chances of generating an early stop codon increase. Also, efficiency and specificity need to be considered. How efficient a gRNA is, reflects its chances of inducing DSBs, while the specificity describes the possibility of inducing off-targets, meaning DBSs in a region that is not the target sequence. These values need to be evaluated together not favouring one completely over the other (Konstantakos et al., 2022).

1.4.1 Novel approaches to CRISPR for gene editing

One way of using the CRISPR method is through gene activation, or CRISPRa, which is when a gene is overexpressed. Here dCas9 is used, which is dead and inactive Cas9 enzymes, that are mutated and unable to cut DNA (Spencer, 2020). By targeting sequences of gRNA the dCas9 can precisely bind to defined genomic sequences (Hong et al., 2018). Researchers found that although the enzyme was inactive, it was still able to target specific genome sites. For the activation, a ribonucleoprotein (RNP) carries transcriptional activators, to overcome the effect of blocking the transcription caused by dCas9. The intention here is to turn the transcription at the targeted promoter region within the target gene, back on again (Spencer, 2020).

In some cases, it is advantageous to produce transgene-free lines of plants and remove any CRISPR/Cas9 sequences, one of the reasons being to make the genome more stable (Yang et al., 2023). Another reason is that the new regulations will require that no extra DNA is present. In this way, one can avoid that the plants are regulated as GMOs. Some of the approaches used to obtain such transgene plants are time-consuming and require lengthy regeneration and selection steps. Yang et al. (2023) designed transcripts of gRNA and Cas9 which are transported from the roots of transgenic plants onto wild-type shoots, as a form of grafting. The Cas9/gRNA is mobile by having added tRNA-like sequences (TLS) that transport the proteins from the transgenic roots onto the wild-type shoot, causing editing in the wild-type tissue and production of genome-edited seeds, and thereby transgene-free offspring (Yang et al., 2023).

Scintilla et al. (2022) found a way of preserving elite cultivar genotypes in grapevine (*Vitis vinifera*) while still performing precise genetic modifications using protoplasts and CRISPR/Cas. Protoplasts were retrieved from embryonic grapevine calli, which could further be edited at target-specific genes using CRISPR/Cas9 leading to their subsequent regeneration as fully edited grapevine plants. This way edited plants can be regenerated through a single-cell based and DNA-free CRISPR/Cas9 methodology. This is also known as transient expression, which rely on the short-term expression of transgenes without the integration of any new material or transgenic cassettes into the genome of the species genome of interest. Regarding new regulation for gene editing, this is very relevant since no foreign DNA will be introduced utilizing this method (Page et al., 2019).

Another CRISPR method, Multi-knock, is a genome-scale CRISPR toolbox which simultaneously targets multiple gene-family members in *Arabidopsis*, which overcomes functional redundancy and identifies genetically hidden components (Hu et al., 2023). This can enable the discovery of the precise functions of individual genes.

Another similar technique to CRISPR is prime editing, a quite recently developed technology (Anzalone et al., 2019). As with CRISPR, prime editing utilizes a Cas9 protein and a guide RNA but does not create a double-stranded break. Instead, a Cas nickase is used to create a nick

in one of the sequences rather than a break which removes both strands (Scholefield and Harrison, 2021). Removing the need for NHEJ, makes prime editing less error-prone than the CRISPR/Cas9 technology and the risk of mismatches is reduced. Another technique, base editing, can also perform edits without DBS, however as of now this method can only be used for transition mutations (C→T, G→A, A→G, and T→C) and not transversion mutations (C→A, C→G, G→C, G→T, A→C, A→T, T→A, and T→G) (Anzalone et al., 2019).

Researchers in plant sciences, use CRISPR/Cas9 in breeding programs to improve resistance against plant pathogens after its first applications in plants in 2013(Dort et al., 2020, Zhu et al., 2020). However, there have not been many achievements when it comes to CRISPR/Cas9-editing in forage grasses, although the technology was immediately utilized in crop and model plants (Sustek-Sánchez et al., 2023).

1.4.2 Gateway cloning and Golden Gate assembly

Gateway cloning is a two-step recombination process that maintains the reading frame while allowing the transfer of DNA fragments between vectors. It works as an ubiquitous promoter, targeting all parts of the organism containing the targeted gene sequence (Akbari et al., 2009). By using a specific donor vector and the gene insert of interest a BP cloning reaction takes place between attP sites flanking the donor vector and attB sites from primers added to the insert. The donor vector will leave a ccdB gene, a toxic protein by-product, and this will in term enhance the cloning efficiency. Excising this gene is crucial, as cells that expresses this gene will not propagate. The BP reaction will generate an entry clone with attL sites, which will be used for an LR reaction together with a destination vector containing attR sites. This construct will be ready for transformation into competent *E. coli* cells and further, into *A. tumefaciens* for the transformation of calli or plant tissue (Hartley, 2003, Chee and Foan, 2015). These principles of Gateway cloning is illustrated in Figure 12.

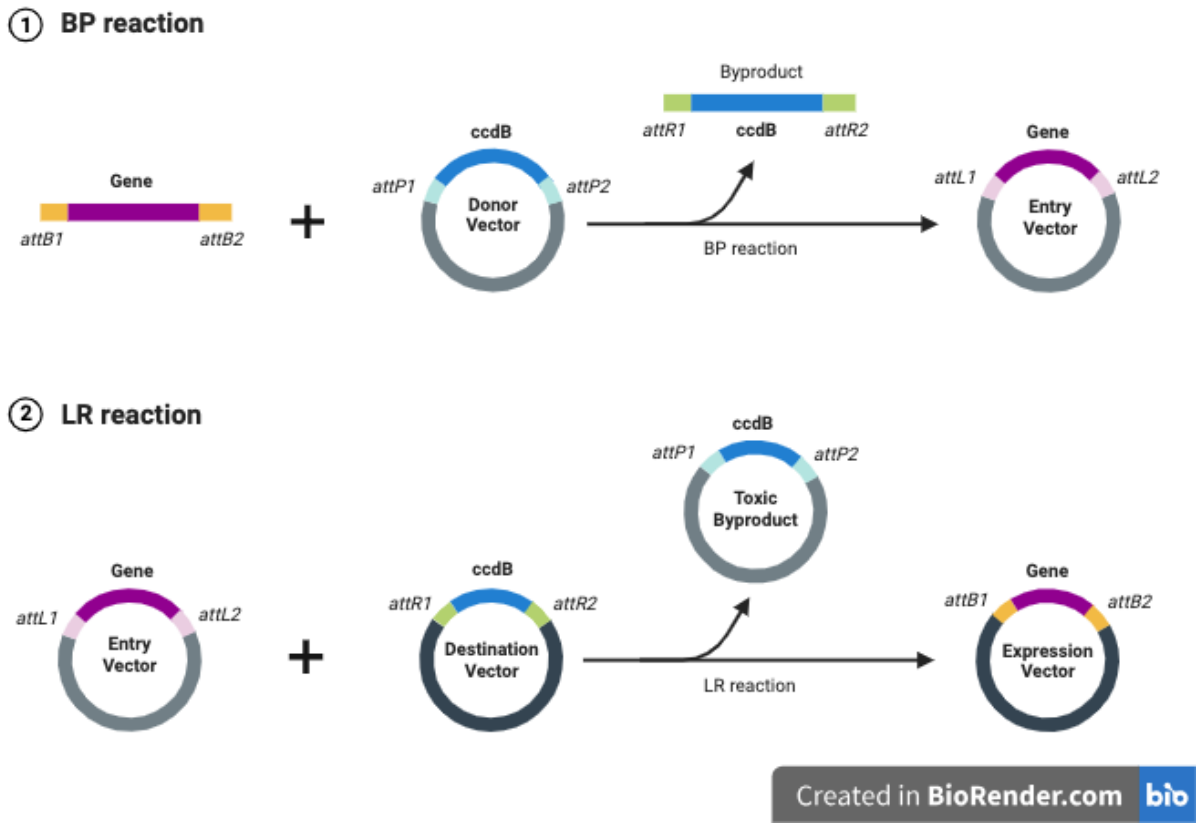

Figure 12. Illustration explaining the principles of Gateway cloning. The BP reaction utilizes attB sites and a donor vector to produce an entry vector. The entry vector together with a destination vector removes the ccdB byproduct through the LR reaction, creating the expression vector. This illustration was created in Biorender, inspired by the Gateway illustratition by Snapgene (snapgene.com)

Golden Gate assembly uses ligase, usually T4 ligase (Potapov et al., 2018), and type IIS restriction enzymes to assemble multiple DNA fragments in a single step into a vector in a linear defined order (Marillonnet and Grützner, 2020). Type IIS restriction enzymes cut DNA at specific sites located outside their recognized DNA sequences (Marillonnet and Grützner, 2020).

1.5 Efficient transformation technologies

To utilize CRISPR/Cas9 as a tool, one needs to have well-established transformation protocols for efficient gene editing. Plant tissue culture is the basis for transformation.

1.5.1 Plant tissue culture

Plant tissue culture is defined as the aseptic growth and development of plant cells, seeds, organs, explants, tissues or protoplasts in defined chemical and physical conditions, *in vitro*

(Thorpe, 2007). Most plant transformation systems rely on this fundamental capability to regenerate plants from isolated cells or tissue, and this plasticity for cell differentiation is what allows plants to alter their metabolism, growth, and development to best suit their environment. There are several utilizations of tissue culture, such as the production of pathogen-free plants, mass propagation of specific clones, genotype modification and plant regeneration after transformation and more.

1.5.1.1 Different types of plant tissues and calli

When producing plant tissue cultures, different types of calli can be produced. Ratios of exogenously added hormones in the nutrient media will also affect what type of calli is generated. Cytokinin and auxin will induce calli in several species, and a high auxin-to-cytokinin ratio will produce root generation, a high cytokinin-to-auxin ratio produces shoot generation, while an intermediate ratio of the two will promote callus induction (Ikeuchi et al., 2013).

There are different types of calli. Meristematic calli, which derive from plant meristems after being cultured in nutrient media containing the auxin 2,4-dichlorophenoxacetic acid (2,4-D) and produce a mass of undifferentiated cells (Torné and Santos, 1990). Somatic calli, which can derive from any part of the plant and consist of dedifferentiated plant cells. These cells have lost their specific characteristics making them able to divide and generate masses of undifferentiated cells (Kallerhoff and Alibert, 2003). Embryonic calli, which come from embryonic plant tissue like seeds or zygotic embryos, possess the mechanism of regenerating whole plants from somatic embryogenesis (Rose and Song, 2017). Here, the cells go through morphological and developmental changes to form embryo-like structures.

1.5.2 Protoplast and *Agrobacterium*-mediated transformations

1.5.2.1 Protoplasts

Protoplasts are cells, without a cell wall that is bound by a plasma membrane but still have all other features and activities of other plant cells. A commonly used approach to study functional genomics in plants are through transient expression of inserted DNA in protoplasts (Davis et al., 2020). Transformation with protoplasts entails isolating, culturing, and merging protoplasts from higher plants to produce new plants. They can be isolated to

remove the cell wall either mechanically or enzymatically, where the latter is the most effective and utilized. One of the reasons protoplast transformations are useful is due to the fact the cell wall is missing, making it easier to transfer genes without cell wall interference, as well as protoplasts being good model systems for studying transformation and recombinant DNA expression and other cellular events (Davis et al., 2020).

A successful method for achieving transient expression and DNA transfer in protoplasts is PEG-mediated transformation. Polyethylene glycol-mediated transformation can promote the uptake and binding of DNA to protoplasts, at an alkaline pH (Davis et al., 2020, Sharma et al., 2022). The PEG method is easy to use without the need for special equipment, however, it is mostly suitable as a rapid screen for transient expression rather than for stable, routine transformations of plants (Davis et al., 2020). An efficient protoplast regeneration protocol in *Arabidopsis* could be completed in 15 weeks and could therefore be used for several purposes such as studying cell proliferation, cell wall biogenesis, and *de novo* shoot and root regeneration, compared to other protocols taking 6 months (Jeong et al., 2021).

An important aspect of protoplast transformation is the source tissue from which they derive and can be significant for successful regeneration. This entails the tissue itself, the genotype, growth conditions, or what organ the protoplasts derive from (Reed and Bargmann, 2021).

1.5.2.2 Agrobacterium-mediated transformations

Transformations using *Agrobacterium* are favourable compared to other transformation methods like protoplast transformation, the reason being that obtaining single copy integration of the DNA to the host genome is easier (Poudel, 2021).

To deliver the gene constructs into the calli, a high throughput transformation procedure with *Agrobacterium tumefaciens* for perennial ryegrass*,* described by Bajaj et al. (2006), will be utilized in the present study. *Agrobacterium* is a gram-negative soil-borne bacteria that causes crown gall disease on the roots of plants. It is commonly used for the insertion of genes and CRISPR/Cas9 constructs when gene-editing plants, since it contains a Tumour

Inducing plasmid (Ti-plasmid) where the desired gene or sequence can be inserted, working as a natural vector system. Strains of *Agrobacterium* used for transformations contain binary vectors with cloning sites as well as the separate helper Ti-plasmid. The Ti-plasmids encode virulence proteins (Vir-proteins), which are necessary for transferring the T-DNA from the plasmid to the plant genome. This pathway is induced by a phenolic compound produced by injured plants, called acetosyringone. When transforming calli, acetosyringone will have to be externally added to achieve the same cascade of events (Niazian et al., 2017).

1.6 Hypothesis/aim of the thesis

The main aim of the thesis is to knock out the *VRN1* and *VIN3* genes to establish an efficient transformation protocol for perennial ryegrass. This will be done by developing CRISPR/Cas9 constructs for knocking out two vernalization genes, *VRN1* and *VIN3* in 2 different genotypes of perennial ryegrass; Fagerlin-201, which can flower without vernalization, and Fagerlin-204, which requires vernalization to flower. Overexpression constructs for the two genes will also be made, to compare material with knocked out genes with overexpressed gene material.

As of now, there are no established protocols for performing CRISPR/Cas9 in perennial ryegrass, and not many published articles describing the methods for this monocot. The present thesis is mainly focusing on establishing protocols for transformation of perennial ryegrass calli, and to design and confirm CRISPR/Cas9 constructs with gRNAs that can knock out these two specific vernalization genes.

Specifically, the CRISPR/Cas9 Golden Gate method for knockouts, and Gateway cloning for the overexpression lines will be used. As part of my thesis, I will spend 1 month at the collaborators lab at TalTech University, Tallinn, Estonia learning and designing gene constructs. The methods established in this thesis will be further used to establish a CRISPR/Cas9 system in perennial ryegrass.

In this thesis, the aim is also to produce meristematic, somatic, and embryonic calli from perennial ryegrass and establish methods for producing such calli from the two genotypes of perennial ryegrass.

2. Materials and methods

All plant materials used for the experiments come from the cultivar “Fagerlin” of perennial ryegrass. Within Fagerlin, we found two genotypes Fagerlin-201 and Fagerlin-204, which differ in vernalization requirement for flowering (Islam, 2015). Fagerlin-201 manages to flower without vernalization, however, Fagerlin-204 requires vernalization to flower. The difference is caused by variation in the vernalization genes, needed to initiate flowering in perennial ryegrass.

All experiments were conducted at the Centre for Plant Research in Controlled Climates (SKP) at the Norwegian University of Life Sciences, in Ås, Norway.

Figure 13 shows a schematic illustration representing every aspect of the methods utilized for this thesis.

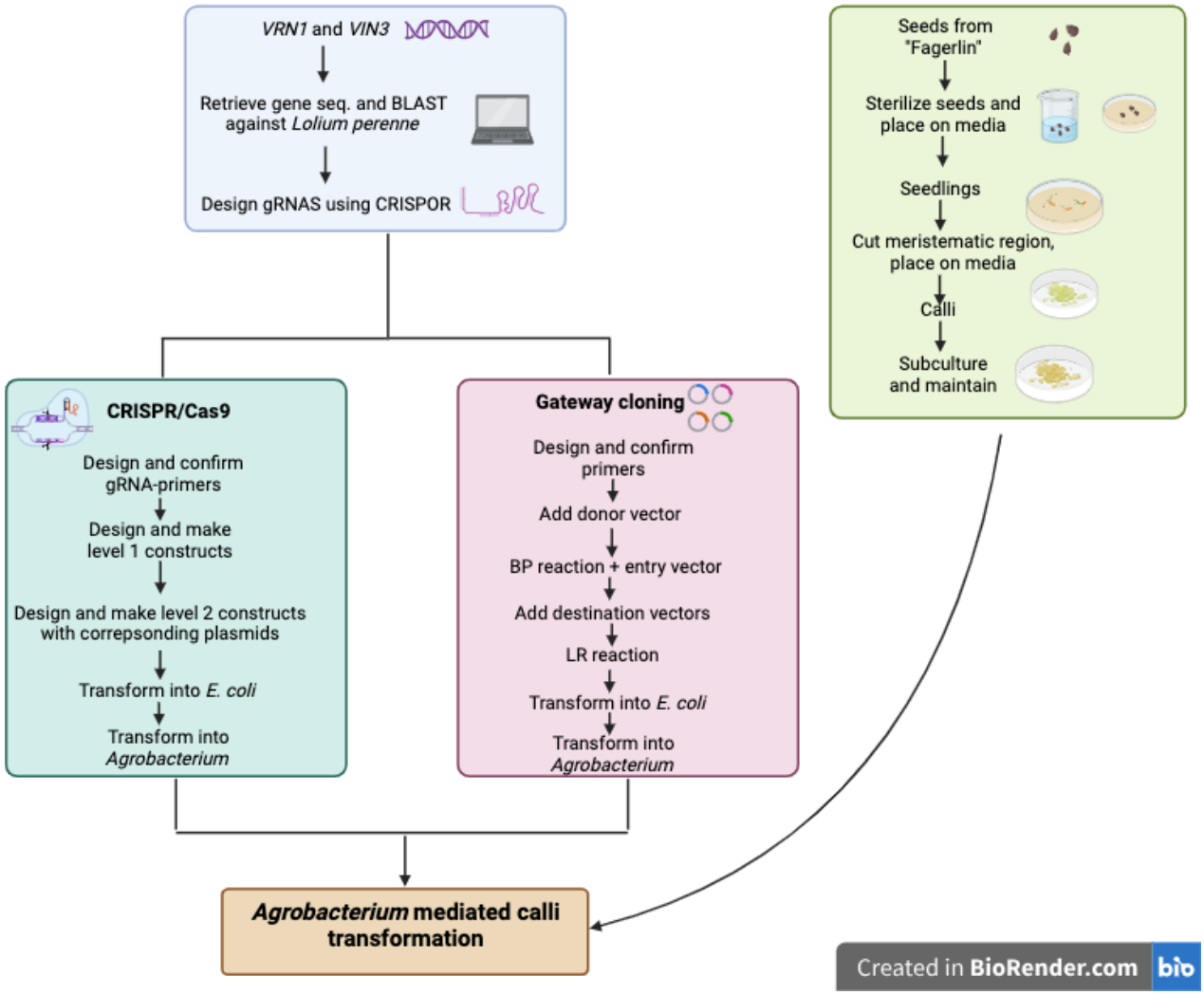

Figure 13. Schematic illustration showing every part of the methods involved in this thesis. For the VRN1 and VIN3 genes sequences have been retrieved in order to design primers and gRNAs, while also producing and maintaining meristematic calli of the perennial ryegrass cultivar Fagerlin. For CRISPR/Cas9 knockout and Gateway cloning, there are some different steps before both constructs can be transformed into E. coli, then further into Agrobacterium before transforming the Fagerlin meristematic calli.

2.1 Plants in growth chambers

Twelve clones of each genotype were placed in growth chambers mimicking short days, at 6 °C with 8 hours of light and 16 hours of dark. The plants were kept there for 6 weeks for vernalization until being placed in growth chambers, mimicking long days with 16 hours of light and 17 °C to induce flowering. The purpose was to produce and collect seeds of different stages to produce embryonic calli.

2.2 Calli production

2.2.1 Meristematic calli

2.2.1.1 Seed based calli

The method of Bajaj et al. (2006) has been modified and used to produce meristematic calli from “Fagerlin” seeds.

Using the method described by Bajaj et al. (2006) perennial ryegrass seeds from the Graminor cultivar “Fagerlin”, were used for induction of meristematic callus. Seeds were first treated with 40% sulfuric acid, containing 18 mL of water and 12 mL of H_2SO_4 for 20 minutes. Further, the seeds were washed for 1 hour in cold running water, before being incubated at 56 °C for 15 minutes. Seeds were surface sterilized with a 25 mL solution containing 1.25 % bleach and 10 μL Tween 20, kept for 20 minutes. Then, the seeds were washed approximately 5-6 times with sterile water, before being dried on sterile filter paper and placed on basal MS media. The seeds were kept in the cold for 3 days before being put in darkness for 6 days.

The plain Murashige and Skoog half MS media contains 3 % sucrose, with a pH adjusted to 5.7, and was solidified with 0.3% phytagel (Bajaj et al., 2006).

Seedlings produced after 1 week in darkness, were exposed to light for 2 days before being divided into 3 regions, with region I and II being closer to the seed and meristematic region, and region III being the outermost part of the seedling, mainly leaves. The workflow from Bajaj et al. (2006) can be seen in Figure 14. Bajaj et al. (2006) describe cutting the seedling 1 cm away from the meristematic region and further dividing it longitudinally before being placed on callus induction media. However, this was not possible with the material produced by the “Fagerlin” seeds after following the protocol. Instead, each region was slightly damaged to more easily take up nutrients from the media.

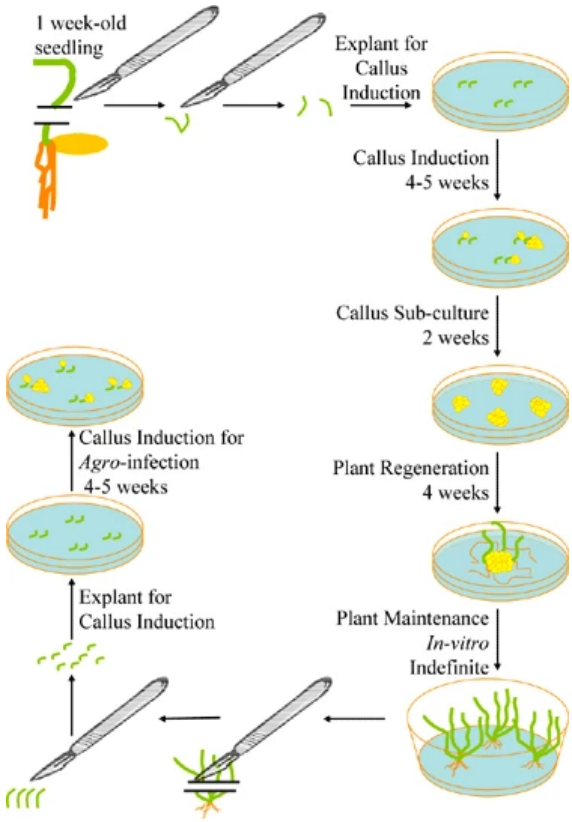

Figure 14. Illustration of steps for callus induction protocol from Bajaj et al. (2006)

The meristematic regions from the seedlings were placed on plates with plain MS media and cultured in the dark for 4-5 weeks at 24 ± 2 °C. Further, the small calli from these regions were placed on MS media containing 22.6 μM of 2,4-dichlorophenoxyacetic acid (2,4-D) and a pH of 5.7, before being placed in the dark for 3-4 more weeks. Then, the calli were placed on MS media containing 3 mg 1^{-1} 2,4-D and 0.05 mg 1^{-1} BAP (Liu et al., 2006), and some were placed in light and some in dark for 2 weeks in 24 +- 2 °C.

Leaves taken from the clones themselves were also used to produce somatic calli. These were surface sterilized with 5 % bleach solution for 10 minutes, before being washed 6 times with sterile water. Further, the leaves were cut in both ends of the leave into smaller pieces for more exposure, before being placed on MS media and kept in the dark for 6 weeks.

2.2.1.2 Meristem based calli

Meristems were retrieved from genotypes Fagerlin-201 and Fagerlin-204 to produce meristematic calli for transformation protocols.

Meristems were collected by cleaning and peeling tillers from the two genotypes removing any dead leaves and soil, before adding them to a 50/50 EtOH solution. Further, the meristems were placed in a 50/50 bleach solution on a roller for 1 hour, before being washed with sterile water and placed on autoclaved filter paper before being dissected. Dissections were performed by cutting longitudinally and the tissues were placed on MS media containing 22.6 μM of 2,4-D, in darkness for 3-4 weeks.

Further, the meristems were moved to MS media containing 3mg 1^{-1} 2,4-D and 0.05 mg 1^{-1} BAP (Liu et al., 2006), and some were placed in light and some in darkness for 2 weeks at 24 +- 2 °C.

2.2.2 Embryonic calli

Possibly fertilized seeds were retrieved from perennial ryegrass plants from a research field at Vollebekk Research Farm, Ås Norway. The seeds were dissected to retrieve embryos to produce embryonic calli.

2.3 Developing overexpression constructs

In this project, Gateway cloning was used for producing an overexpressor line for the *VRN1* and *VIN3* genes.

Based on the template sequence for the *VRN1* and *VIN3* genes (Kovi et. al, unpublished), Gateway primers containing attB1 and attB2 attachment sites, flanking the 18 first and last bases of the sequence were designed.

Using the Qiagen RNeasy plant mini kit, RNA was extracted from plant material from both genotypes. Further, this RNA was used to perform cDNA synthesis. A master mix was prepared by mixing 5X VILO Reaction Mix with 10X Superscript enzyme mix, nuclease free H_2O and the RNA samples, according to Table 1. The samples were incubated at 25 °C for 10 minutes, 40 °C for 1 hour and 85 °C for 5 minutes.

Table 1. Overview of reagents and volumes used for cDNA synthesis.

Reagents	Volumes
5X VILO reaction mix	4 µL
10X superscript enzyme mix	2 µL
Nuclease free H_2O	According to RNA concentration
RNA samples	500 ng
	= 20 µL

To amplify the primers, a gradient PCR program was run in order to find the most suitable annealing temperature. Further, a PCR program with an annealing temperature of 70 °C was performed with the primers with attB overhangs and a master mix of the different cDNA.

Table 2. Overview of volumes and annealing temperatures used for gradient PCR.

Gradient PCR annealing temperatures							
58 °C	58.9 °C	60.5 °C	62.9 °C	65.8 °C	68.4 °C	70 °C	71 °C
Reagents				**Volumes (µL)**			
Phusion Mastermix				20			
***VRN1*_forward primer**				4			
***VRN1*_reverse primer**				4			
Nuclease free H_2O				10			
cDNA template mix				2			
				= 40 µL = 5 µL in 8 PCR tubes			

Table 3. PCR program as well as volumes and reagents for amplification of the VRN1 primers.

Reagents	Volumes	Program	
Phusion Mastermix	20 µL	98 °C – 1 minute	30 cycles
Forward primer (*VRN1*)	4 µL	98 °C - 10 seconds	
Reverse primer (*VRN1*)	4 µL	70 °C – 10 seconds	
Nuclease free H2O	10 µL	72 °C – 30 seconds	
cDNA master mix	2 µL	72 °C – 10 minutes	
	= 40 µL	4 °C - infinity	

Further, a new PCR run following the same PCR program as the one described in Table 3 was conducted after confirming the primers on a 1% gel. The new PCR included the PCR product from the previous run together with the attB primers.

For the BP cloning reaction, a PCR product containing the samples and attB primers was used when following the Invitrogen protocol for BP cloning. This was done to create an entry clone with the empty pDONR207 vector, shown in Figure 15 (SnapGene® software (snapgene.com). Using the PCR product, the empty vector, BP clonase enzyme mix, and TE buffer, the solution was incubated in room temperature for 1 hour before being transformed into top 10 chemo competent *E. coli* cells (Invitrogen) and incubated on ice for 30 minutes. Further, the cells were subjected to heat shock at 42 °C for 30 seconds and incubated with shaking for 1 hour at 37 °C. Further, the cells were plated on LB gentamycin plates and incubated overnight.

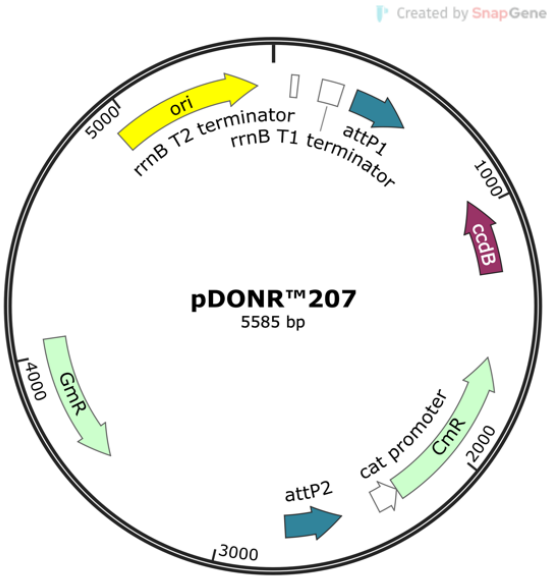

Figure 15. The pDONR207 entry vector. Illustration created by Snapgene (www.snapgene.com/resources)

Table 4. Overview of reagents and volumes for BP reaction.

Reagents	Volumes (µL)
PCR product (*VRN1/VIN3* + attBI)	2
pDONR207	1
BP clonase enzyme mix w/buffer	2
TE buffer	5
	= 10 µL

Further, the transformants from the BP cloning were analysed. Randomly selected colonies were inoculated in liquid LB gentamycin overnight at 37 °C with shaking. A Monarch Plasmid Miniprep kit was used to extract plasmids. Further, the concentrations for each entry clone sample were measured using Nanodrop, before being restricted with the BpiI restriction enzyme incubating at 37 °C for 1 hour. The entry clone samples were confirmed on 1% gels.

One of the obtained restricted positive samples was diluted to a final concentration of 100 ng/µL with the pDONR207 primers and sent for Sanger sequencing at Eurofins Genomics, Germany.

After confirming a successful entry clone, an LR recombination reaction was performed following the Invitrogen protocol, between the entry clone, and the two destination vectors pMDC32 (Gangadhar et al., 2016) and pANIC6A (Mann et al., 2012). 5 µL of LR reaction samples with the two destination vectors were added to top 10 chemo-competent *E. coli* cells (Invitrogen) and was incubated on ice for 30 minutes before being heat shocked at 42 °C for 30 seconds. 500 µL of plain liquid LB media was added to the cells which were incubated at 37 °C for 1 hour with shaking. The tubes were centrifuged for 2 minutes at 10,000 RPM and 500 µL was removed. The remaining cell contents were mixed in the tubes and plated on LB kanamycin overnight at room temperature.

Table 5. Reagents and volumes for LR reaction.

Reagents	Volume (µL)
pDONR207_ entry clone	2 (150 ng/µL)
TE buffer	12
LR clonase	4
Either pMDC32 or pANIC6A	1
	= 19 µL

Further, the cultured growth was inoculated by selecting random colonies from the plates with the different destination vectors and added to 4 mL of liquid LB kanamycin media at 37 °C overnight with shaking.

To confirm the destination vectors, plasmids from the inoculated samples were extracted using the GeneJET miniprep plasmid kit from Thermofisher company. The plasmids were restricted using BamHI at 37 °C for almost 3 hours. The samples were run in 2% gel and confirmed by looking for the right band size found using the sequence analysis tools. molecular biology software SerialCloner.

For *Agrobacterium* transformation, 5 µL of each destination vector was added to two separate tubes of chemically competent GV3101 *Agrobacterium* (pMP90) cells. These were incubated on ice for 5 minutes before being added to liquid N_2 for 5 minutes. Further, the cells received heat shock at 37 °C for 5 minutes, before 1 mL of liquid LB media was added.

The cells were placed at 28 °C for 3 hours with shaking, before being plated on LB rifampicin, gentamycin, and kanamycin (RGK) plates and incubated in 28 °C for 2-3 days.

To confirm that the *Agrobcaterium* contains the binary vectors, a re-transformation into *E. coli was* been done. Colonies from each destination vector were inoculated overnight in liquid LB RGK. 3 mL of the solutions were used for extracting plasmids with the GeneJET miniprep plasmid kit. Further, 30 µL of plasmid product was used to transform into top 10 chemo-competent *E. coli* cells (Invitrogen), incubated on ice for 30 minutes, and heat shocked for 30 seconds at 42 °C. Further, the cells were placed in ice for 1 minute before 500 µL of plain liquid LB was added. The solution was incubated for 1 hour at 37 °C with shaking, before being centrifuged. 500 µL of the supernatant was removed, and the remaining pellet was mixed and plated onto LB kanamycin plated and incubated at 37 °C over night.

Random colonies were inoculated in 3 mL liquid LB kanamycin at 37 degrees overnight with shaking. The inoculated colonies were pelleted down, and plasmids were extracted using the GeneJET miniprep plasmid kit. Plasmids were restricted with BamHI at 37 °C overnight.

Agrobacterium transformation was confirmed when observing the same band sizes and the number of bands as when confirming the transformation of destination vectors.

After confirming *Agrobacterium* transformation, glycerol stocks of the destination vectors in *Agrobacterium* and *E. coli* were prepared. 500 µL of 50% glycerol was added with 500 µL of each of the samples, adding them to liquid N_2 before being placed at -80 °C.

Transformation of calli with overexpression constructs

Pipette tips with glycerol stocks of the pANIC6A_*VRN1* and pMDC32_*VRN1* constructs were streaked out on LB RGK plates and incubated at 28 °C for 2 days. 2 colonies from each construct were inoculated in liquid LB RGK at 28 °C overnight.

Further, 1 mL from one of these inoculated colonies from each construct was reinoculated in 100 mL of liquid LB RGK at 28 °C with 230 RPM overnight. The optical density (OD) was measured and diluted to be between 0.6 and 0.8. The solutions were pelleted down at 4,000

RPM for 5 minutes before being washed in basal half MS media containing 1% glucose and 3% sucrose, pH=5.2. Again, the solutions were centrifuged for 5 minutes before being resuspended in the same basal MS media with added 400 µM acetosyringone.

Subcultured perennial ryegrass calli were placed in the solutions shaking at 50 RPM for 1 hour in darkness. Excess liquid was removed before the calli were blotted and placed on autoclaved filter paper onto co-cultivation MS media containing 0.44 µM BAP, 9 µM 2,4-D and 400 µM acetosyringone.

After 4 days, the calli was washed with basal MS several times, to remove any excess growth of *Agrobacterium*, before being dried on autoclaved filter paper. Further, the calli were placed on subculture MS media containing 3% sucrose, 0.44 µM BAP, 9 µM 2,4-D, 94.8 µM hygromycin and 500 mg/L cefotaxime. This procedure was repeated 3 more times with 4 days intervals to ensure that there would be no overgrowth of *Agrobacterium*. Further, the calli were rinsed with 30 µL of Timentin added to the basal MS media, before being placed on subculture MS media containing 500 mg/mL Timentin instead of cefotaxime.

2.4 Developing CRISPR/Cas9 knockout constructs

2.4.1 Designing gRNAs

Four gRNAs for each gene, *VRN1* and *VIN3*, were designed with the following approach. The Basic Local Alignment Search Tool (BLAST) from NCBI (Altschul et al., 1990) was used for finding a full gene sequence of *VRN1* and *VIN3* in perennial ryegrass. Further, the ViroBLAST Lolium_2.6.1 tool (Deng et al., 2007) was used to retrieve an accession number for the correct sequence. The accession number was used to find an annotated sequence in the perennial ryegrass genome, available in the Monocots PLAZA 5.0 database (Van Bel et al., 2021). The annotated sequence was then downloaded into Benchling ([Biology Software]. (2022) retrieved (from https://benchling.com) for further annotations of gRNAs.

gRNAs were designed using the CRISPOR program (Concordet and Haeussler, 2018), evaluating the 4 most suitable gRNAs for each gene based on the parameters efficiency, off-targets and specificity. From these gRNA sequences, the necessary primers were designed. Although 4 gRNAs were designed, not all 4 gRNAs worked when making

constructs. Therefore, only 2 gRNAs were further utilized for designing and making the constructs and transforming the calli.

2.4.2 Designing and confirming gRNA primers

The approach for knock-out of the *VRN1* and *VIN3* genes is mainly based on the pipeline described by Grützner et al. (2021). For this pipeline, plasmids from the MoClo (Weber et al., 2011) and zCas9i (Grützner et al., 2021) toolkits were utilized. The following was done with the designed gRNAs for both *VRN1* and *VIN3*.

CRISPR primers were designed, following the description in Figure 16 below, by adding the sequences from previously designed gRNAs together with the critar-overhangs. The primers for *VRN1* were tested together with the empty pAGM9037 vector.

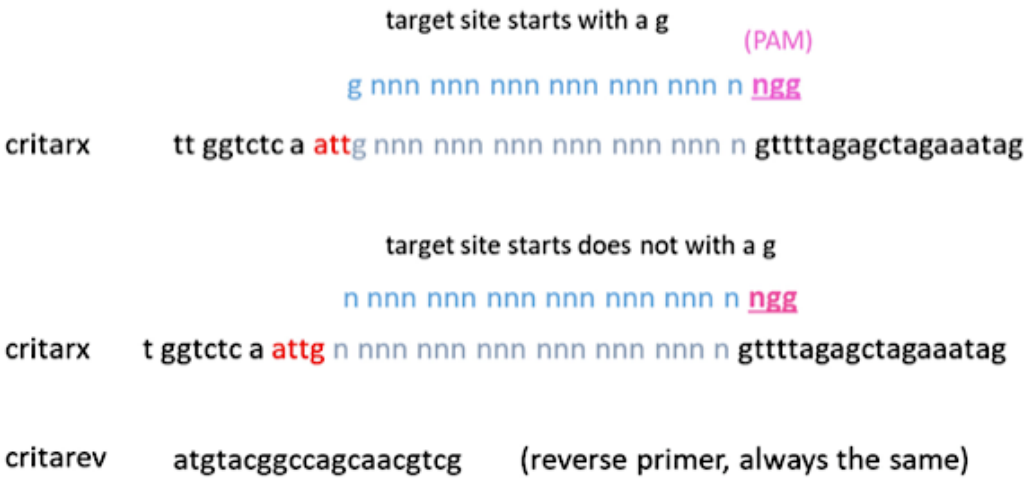

Figure 16. The approach for designing primers follows the method from Grützner et al. (2021), the reverse primer is the same for every construct. The primer design differs depending on wheter the target site starts with a G or not.

A solution with Phusion Mastermix, nuclease free water, critarev primer, template mix of pAGM9037 (18 µL NF H2O + 2 µL pAGM9037 plasmid), and each of the gRNA primers for *VRN1* was run in the PCR program with 30 cycles, is shown in Table 6.

Table 6. Overview of reagents, volumes and PCR program for gRNA primers.

Reagents	Volumes (µL)	Program	
Phusion Mastermix	5	98 °C – 1 minute	30 cycles
pAGM9037 template mix	(2 µL pAGM9037 + 18 NF H2O = 20 µL)	98 °C - 10 seconds	
	1		
CRITAREV primer	1	68 °C – 10 seconds	
Nuclease free H2O	2	72 °C – 15 seconds	
Forward crita_gRNA primers	1	72 °C – 10 minutes	
	= 10 µL	12 °C - infinity	

Further, a 1% gel was run to confirm band sizes and they were as expected: 8,407 bp for the pAGM9037 vector and 630 bp for gRNA primers. After confirming band sizes, all gRNA primers were amplified to get more strands of each product following the same PCR program as previously mentioned, but with 35 cycles.

Table 7. Reagents, volume and PCR program for amplification of gRNA primers

Reagents	Volumes (µL)	Program
Phusion Mastermix	25	98 °C – 1 minute
pAGM9037 template mix	1	98 °C - 10 seconds
CRITAREV primer	2,5	68 °C – 10 seconds
Nuclease free H2O	19	72 °C – 15 seconds
Forward crita_gRNA primers	2,5	72 °C – 10 minutes
	= 50 µL	12 °C - infinity

2.4.3 Generating level 1 constructs

The amplified primer products were then cloned into a level 1 MoClo vector corresponding to gRNA1 and 2, which for gRNA1 is pICH47751 and for gRNA2 is pICH47761. They were added together with a promoter, pAGM38869, using BSaI and ligase for restriction ligation following the protocol from the MoClo Kit (Weber et al., 2011).

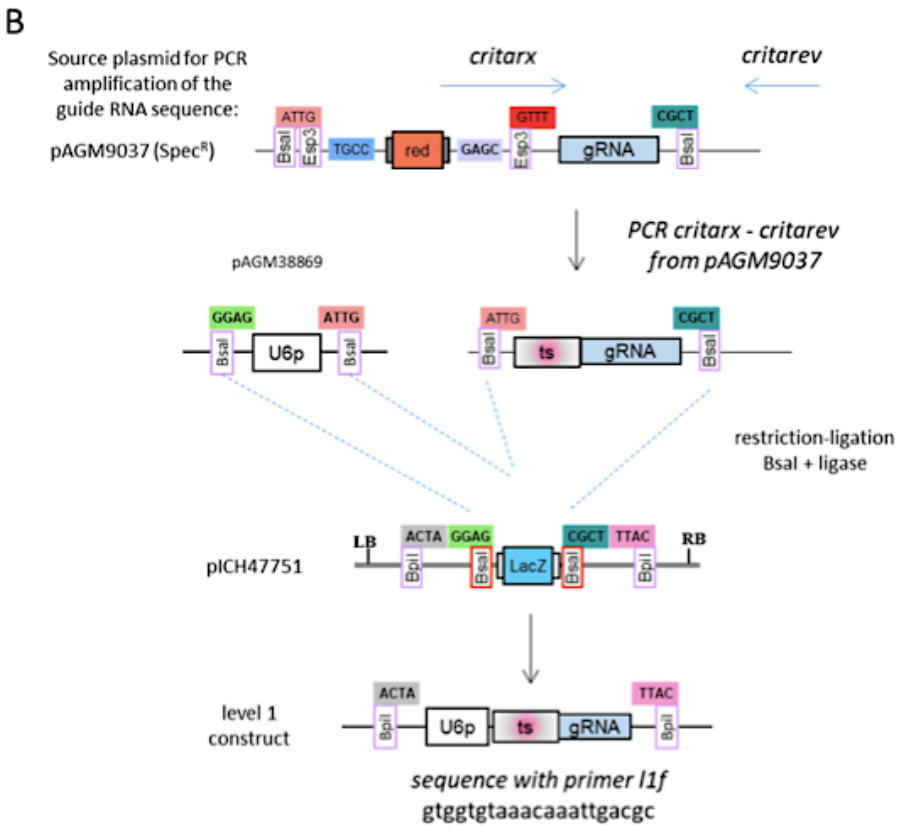

Figure 17. Approach for designing level 1 constructs with gRNAs, adding a U6 promoter from the pAGM38869 plasmid (Grützner et al., 2021) together with the gRNA primer into a pICH plasmid (Weber et al., 2011) respectively of whether the gRNA construct is number 1 or 2, taking the place of the LacZ-promoter making it possible to do blue-white selection. Successfully transformed constructs will be sequenced with the l1f primer (Grützner et al., 2021).

Table 8. Reagents, volumes and program for restriction-ligation of level 1 constructs with gRNA1 and 2.

Reagents	Volumes (µL)	Program – Restriction – Ligation
pICH47751/47761	1	37 °C – 5 hours
pAGM38869	0,5	50 °C – 5 minutes
10x T4 DNA ligase buffer	1	80 °C – 10 minutes
T4 DNA ligase	0,5	12 °C - infinity
BsaI-HFV2	1	
NF H_2O	5	
Amplified gRNA primer	1	
= 10 µL		

Since the restriction-ligation was performed overnight, an additional step of 5 minutes at 60 °C was performed before continuing. The 10 µL of each sample was added into *E. coli* competent cells (Invitrogen), kept on ice for 30 minutes before being given a heat shock at 42 °C for 30 seconds. Further, the cells were placed on ice for 5 minutes before adding 950 µL of plain liquid LB media, shaking at 37 °C for 1 hour. The samples were then pelleted down, the 950 µL removed and the remaining 25 µL were plated on Ampicillin + 20mg/mL X-gal LB plates and incubated at 37 °C overnight for blue-white selection. If the reaction was positive, white colonies would grow, as the gRNA construct would have taken the place of the LacZ promoter yielding blue colonies, as seen In Figure 17.

Further, white colonies from each plate with gRNA level 1 constructs were inoculated overnight at 37 °C in 6 mL of liquid LB media with ampicillin. The GeneJET Miniprep kit was then utilized to extract plasmids from the inoculated colonies, before performing restriction digestion with BpiI at 37 °C for 1 hour.

Table 9. Reagents and volumes for the restriction digestion with BpiI on each pICH/gRNA plasmid

Reagents	Volumes (µL)
BpiI	0,5
10 x buffer	2
NF H_2O	15
pICH/gRNA plasmid	2,5
	= 20 µL

The samples were run on a 2% gel. When running the *VIN3* gRNAs, the pICH47772 plasmid was added as a positive control and nuclease-free water as a negative control. After confirming band sizes, all samples were sent for Sanger sequencing to Eurofins, Germany, together with the *l1f* primer, seen in Figure 16.

2.4.4 Generating level 2 constructs

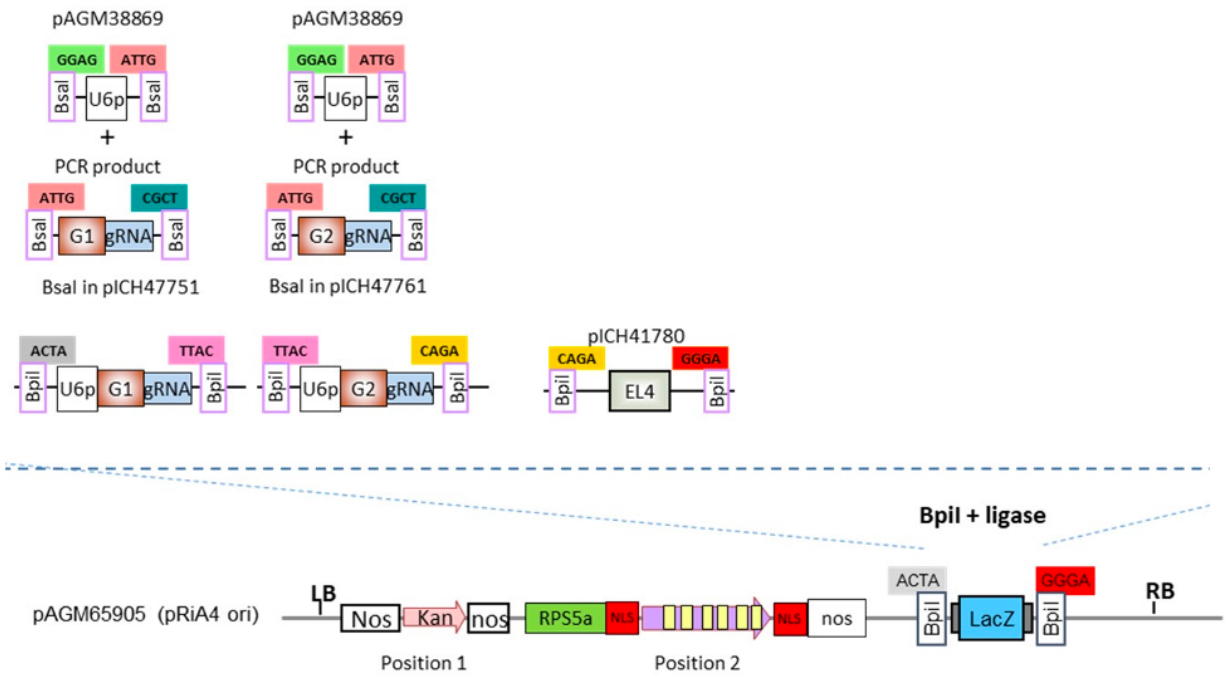

Figure 18. Illustration showing how level 2 constructs, using 2 gRnAs are designed. The level 1 constructs with the correct pICH-plasmid corresponding to each gRNa are ligated together with the pAGM38869 construct and the pICH41780 end-linker. Using restriction ligation with BpiI, the constructs replace the LacZ promoter in the level 2 vector pAGM65905.

After confirming level 1 vectors with the *l1f* primer, restriction ligation with BpiI was performed with the 2 gRNA_pICH vectors together with the end linker corresponding for the use of 2 gRNAs, and the pAGM65905 level 2 vector. This is seen in Figure 18 corresponding to the figure from Grützner et al. (2021), following the program from Weber et al. (2011).

Table 10. Reagents and volumes for restriction ligation for constructing level 2 vectors.

Reagents	Volumes (µL)
pAGM65905	1
pICH41780	1
10x T4 DNA ligase buffer	2
T4 ligase	1
BpiI	1
NF H_2O	12

pICH47751_gRNA1	1
pICH47761_gRNA2	1
	= 20 µL

Further, the two level constructs for *VRN1* and *VIN3* were transformed into top10 chemo-competent *E. coli* cells (Invitrogen), kept on ice for 30 minutes, before being given a heat shock at 42 °C for 30 seconds. Further, the cells were placed on ice for 5 minutes before adding 950 µL of plain liquid LB media, shaking at 37 °C for 1 hour. The samples were then pelleted down, 950 µL was removed and the remaining ~100 µL were plated on Kanamycin + 20mg/mL X-gal + IPTG LB plates and incubated at 37 °C overnight for blue-white selection. White colonies were inoculated in liquid LB media with kanamycin.

After extracting plasmids using the GeneJET Miniprep kit on inoculated colonies, restriction digestion was performed with ApaL1 at 37 °C for 30 minutes.

Table 11. Reagents and volumes for restriction digestion for level 2 samples.

Reagents	Volumes (µL)
ApaL1	1
10 x buffer	1
NF H_2O	4
Level 2 plasmid samples	4
	= 10 µL

A 1% gel was run to check band sizes. Expected band sizes would be 1.9 kb, 3.6 kb and 11.1 kb. Further, the samples were sent for sequencing to Eurofins Genomics, Germany with the level 2 reverse primer.

Further, 1 clone from each gene were transformed into *Agrobacterium* cells, before being plated on LB RGK plates and incubated in room temperature. Colonies from each plate were inoculated overnight in liquid LB RGK and the inoculated colonies were used for extracting plasmids with the GeneJET miniprep plasmid kit to perform *E. coli* a retransformation

reaction. After successful confirmation, glycerol stocks were made of both the *VIN3* and the *VRN1* level 2 constructs.

2.4.5 Transformation of calli using level 2 constructs

The inoculated colonies were further used for the same *Agrobacterium* transformation protocol as for the overexpression constructs. Briefly, 3 mL from the inoculated colonies from each construct was reinoculated in 100 mL of liquid LB RGK at 28 °C with 230 RPM ON. OD was measured and diluted to be 0.6-0.8. The solutions were pelleted down at 4,000 RPM for 5 minutes before being washed in basal half MS media. Again, the solutions were centrifuged for 5 minutes before being resuspended in the same basal MS media with added 400 µM acetosyringone.

Subcultured perennial ryegrass calli were placed in the solutions shaking at 50 RPM for 1 hour in darkness. Excess liquid was removed before the calli were blotted and placed on autoclaved filter paper onto co-cultivation MS media.

After 3 days, the calli was washed several times with basal MS with 30 µL of Timentin added, several times to remove any growth of *Agrobacterium*, before being dried on autoclaved filter paper. Further, the calli was placed on subculture MS media containing 3% sucrose, 0.44 µM BAP, 9 µM 2,4-D, 94.8 µM hygromycin and 500 mg/L Timentin. This procedure was repeated 2 to 3 more times with 4 days intervals to ensure that there would be no overgrowth of Agrobacterium.

3. Results

3.1 Perennial ryegrass genotypes in growth chambers

After treating the plants in both LD (17°C and 16 hrs light) and SD (6 °C and 8 hrs light) for 6 weeks in growth chambers, the plants were moved into greenhouses with a temperature between 18-22 °C and 16 hours of light (long days) for observing the flowering. This did not lead to any production of seeds or flowering.

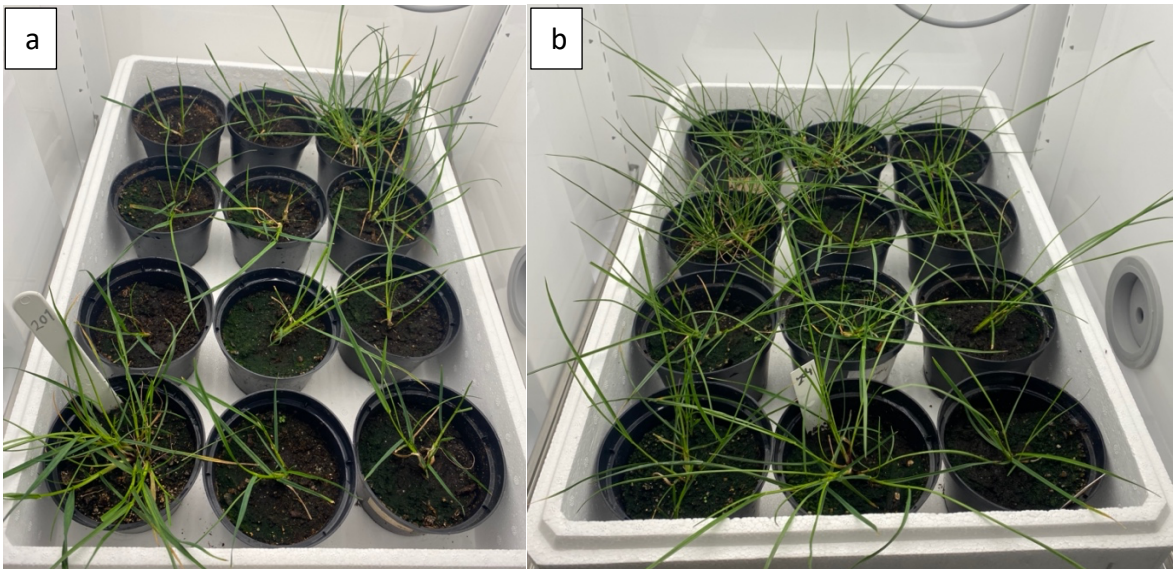

Figure 19. Fagerlin-201 (a) and Fagerlin-204 clones (b) in growth chambers after some weeks.

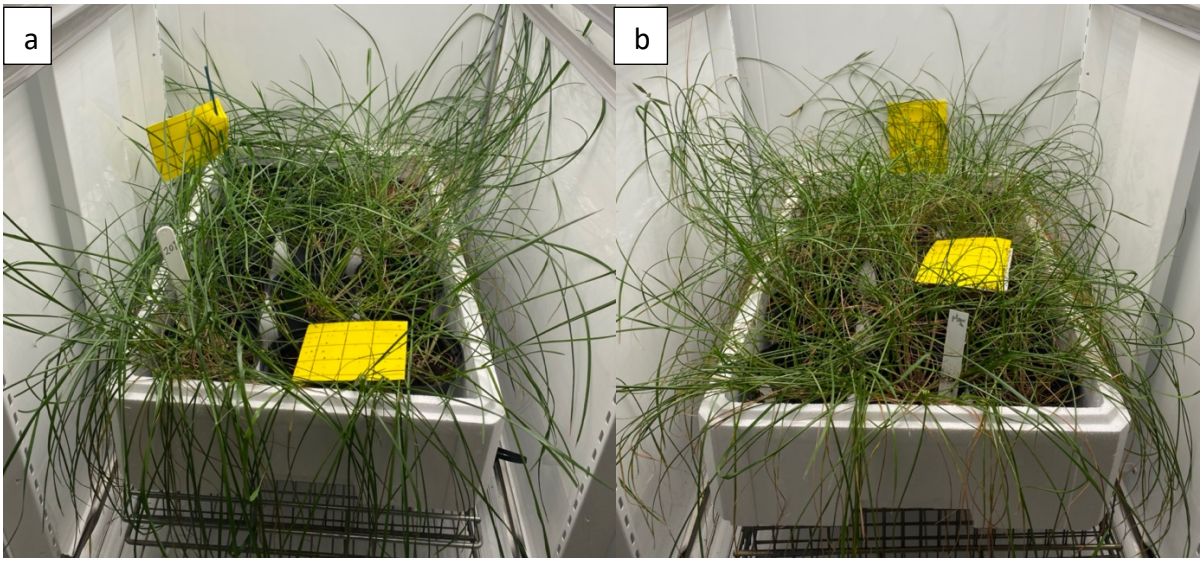

Figure 20. Fagerlin-201 (a) and Fagerlin-204 clones (b) just before being moved to the greenhouse showing extensive growth.

3.2 Seed based meristematic calli

"Fagerlin" seeds that were prepared and washed following the method from Bajaj et al. (2006) produced seedlings. These were cut into regions and placed on plain MS in the dark. The 2 regions closest to the seed and meristem produced small calli, which were further subcultured and maintained on MS media containing the hormones 2,4-D and BAP, before

being used for transformations. Figure 21 shows subcultured calli on media. These were subcultured and maintained for 3-4 months before being utilized for transformations.

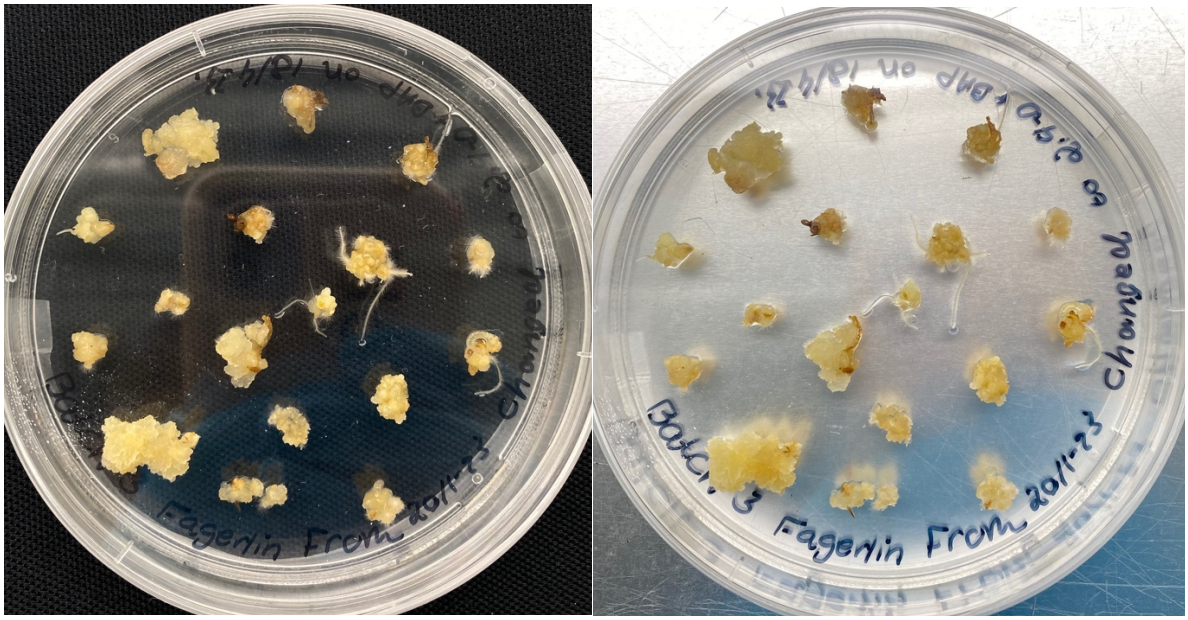

Figure 21. Subcultured calli produced from meristematic regions of Fagerlin seedlings. Bigger calli were separated and all calli were placed on fresh MS media containing 2,4-D and BAP hormones in 2 week intervals.

3.3 Somatic calli

The sterilized leaves from perennial ryegrass placed on MS media in the dark for 6 weeks did not yield somatic calli. Neither did the outermost region from the seed protocol from Bajaj et al. (2006), where the region only consisted of leaf tissue produced by the seedlings.

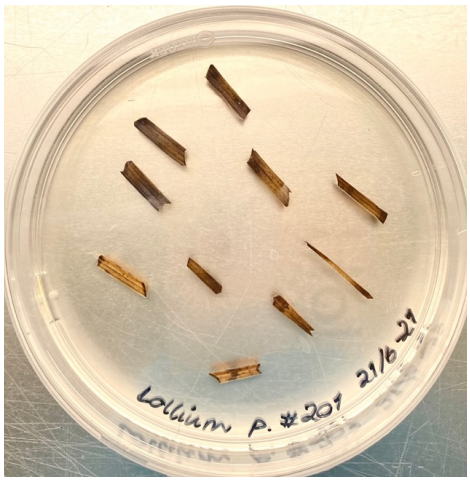

Figure 22. Perennial ryegrass leaves on 2,4-D media after several weeks. No somatic calli were produced.

3.4 Embryonic calli

All seeds found and collected from *L. perenne* plants at Vollebekk fields at NMBU were immature, meaning no mature embryos were dissected and placed on media to produce embryonic calli. Figure 23 shows an immature embryo from perennial ryegrass.

Figure 23. Microscope picture showing an immature embryo and anthers from L. perenne seeds collected from Vollebekk Research Farm, Ås Norway.

3.5 Meristematic calli from Fagerlin-201 and Fagerlin-204 meristems

Meristems collected from several Fagerlin-201 and Fagerlin-204 clones kept in growth chambers and greenhouse did not yield any meristematic calli. Meristems were placed on 2,4-D media and did not produce any calli after several weeks, neither in light nor in the dark.

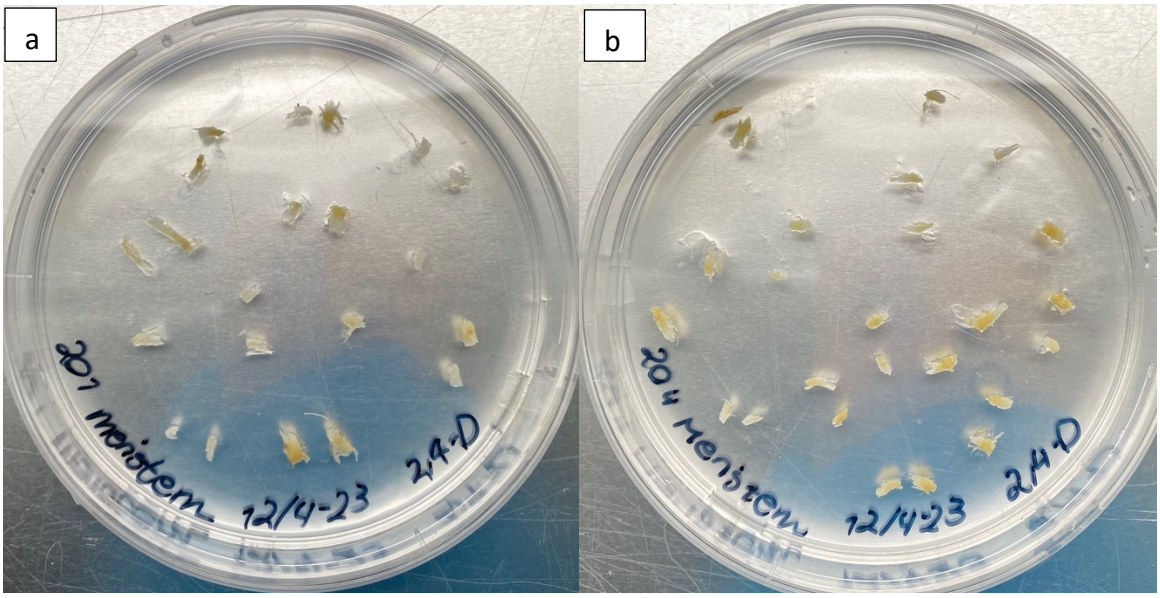

Figure 24. Dissected and sterilized meristems from Fagerlin-201 (a) *and Fagerlin-204* (b) *in 2,4-D media. No calli produced.*

3.6 Overexpression and gateway cloning

3.6.1 VRN1 gene

The amplification of the primers with attB overhangs and the making of an entry clone with the BP reaction with pDONR207 was successful for the *VRN1* primers.

After performing RNA extraction and cDNA synthesis a gradient PCR was run to find the most suitable annealing temperatures for the primers. For *VRN1* this was at 70 °C as seen in Figure 25.

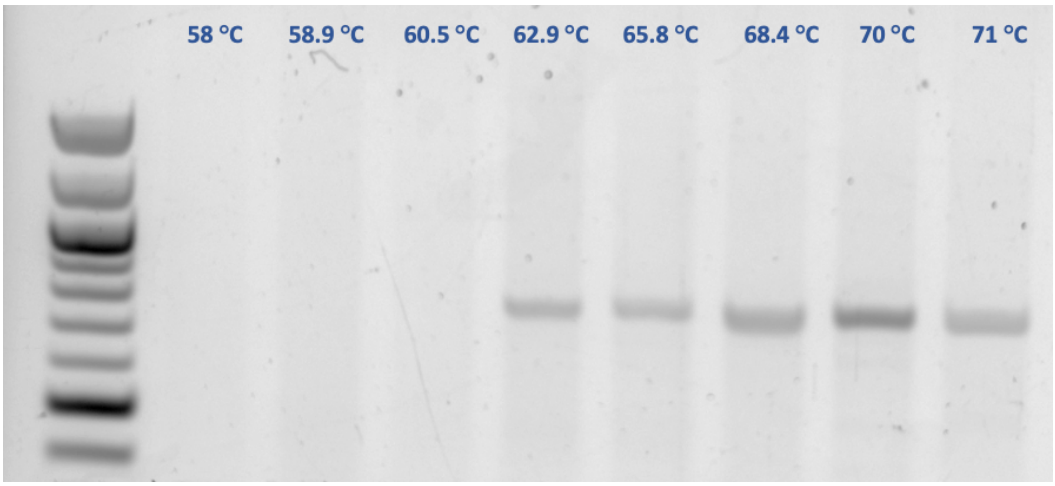

Figure 25. Gradient PCR with VRN1 attB primers. Annealing temperature starting at 58°C up to 71 °C, with the clearest band being at 70 °C

Further, the PCR run with the *VRN1* primers together with the attB primers was confirmed on a 1% gel as seen in Figure 26.

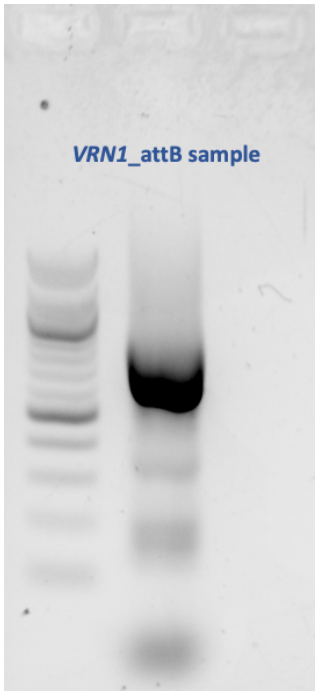

Figure 26. VRN1_attB primer sample run on a 1 % gel.

The BP cloning with the *VRN1*_attB samples from the PCR together with the pDONR207 donor vector was successfully executed. The extracted plasmids used for the BpiI restriction were confirmed on the 1 % gel depicted in Figure 27, showing positive clones.

Figure 27. 3 samples of entry clone VRN1_pDONR207 with attB1 primers restricted with BpiI.

After confirming the entry clone, destination vectors pMDC32 and pANIC6A was added during an LR reaction. The successfully transformed *E. coli* colonies that were inoculated and from which plasmids were extracted, were restricted and confirmed using BamHI. A successful pANIC6A_VRN1 destination vector was expected to have 14.9 kb and 1.1 kb bands. The pMDC32_VRN1 destination vector was expected to have one band at 10.8 kb. This can be seen in Figure 28.

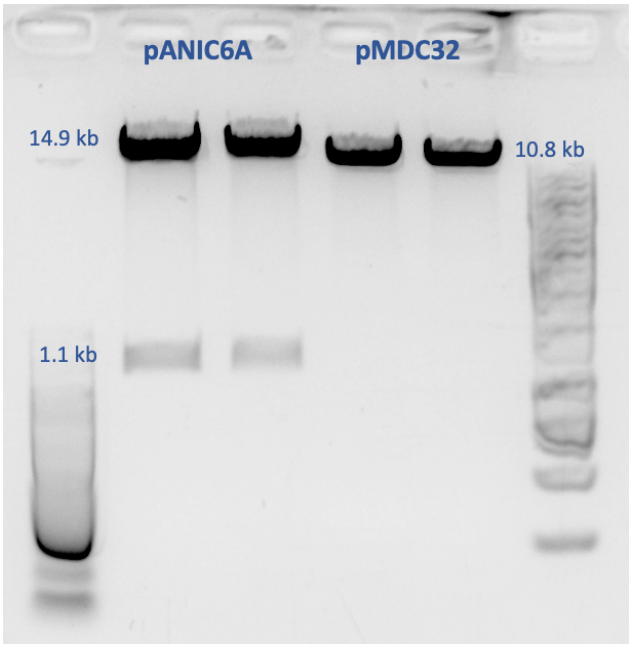

Figure 28. Confirmation of destination vectors with BamHI restriction enzyme. 2 samples of pANIC6A_VRN1 with 2 bands at 14.9 kb and 1.1 kb, and 2 samples of pMDC32 with one band each at 10.8 kb.

Further, the destination vectors were successfully transformed into *Agrobacterium,* retransformed into *E. coli* and confirmed using the same restriction enzyme, BamHI, with the same expected band sizes, and could further be used for calli transformation.

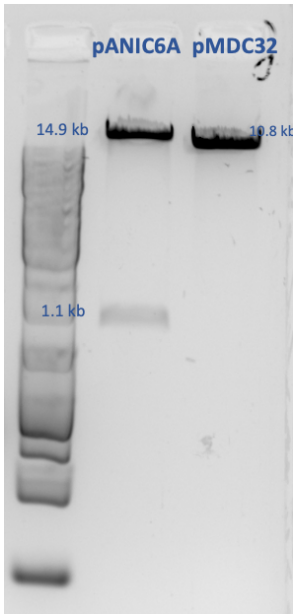

Figure 29. Confirmation of destination vectors transformed into Agrobacterium using BamHI. pANIC6A_VRN1 with band sizes 14.9 kb and 1.1 kb and pMDC32_VRN1 with band size 10.8 kb.

3.6.2 Transformation of calli with overexpression constructs

The subcultured perennial ryegrass calli utilized for transformations showed growth and development of more cells.

Both batches of calli, the ones with the VRN1_pANIC6A and the VRN1_pMDC32 constructs showed growth after changing the media and rinsing the calli with MS basal media with added timentin. Figure 30 depicts calli transformed with each construct, showing calli where the lighter areas represent newly generated tissue on Hygromycin selection medium, produced post-transformation, meaning that the transformations were successful.

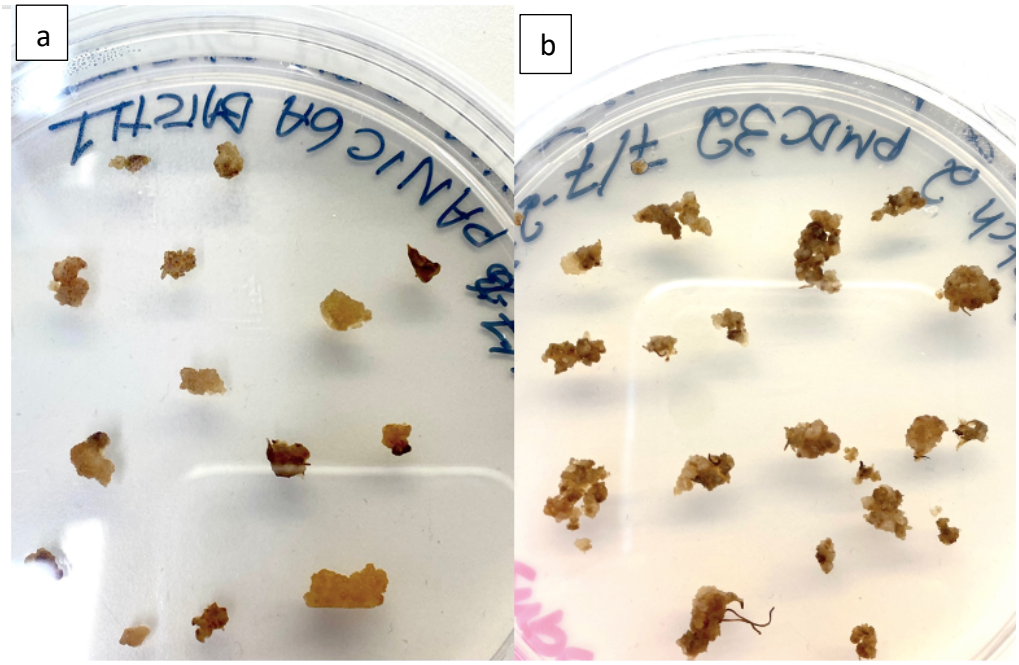

Figure 30. Agrobacterium-transformed perennial ryegrass calli showing growth. Lighter areas on the calli shows where the calli is developing and producing more tissue. (a) Calli transformed with the VRN1_pANIC6A overexpression construct, and (b) calli transformed with the VRN1_pMDC32 overexpression construct.

3.6.3 VIN3 gene

The same method performed for *VRN1* was also applied for *VIN3.* Gradient PCRs were performed with annealing temperatures spanning from 43 °C up to 71 °C. No proper bands were detected. PCR runs were performed both with cDNA samples as well as gDNA samples from the Fagerlin-201 and Fagerlin-204 plant material. The primers for *VIN3* were also redesigned and a new gradient PCR was performed. Figure 31 shows a representation of the results provided by all the different methods applied for the *VIN3* primers.

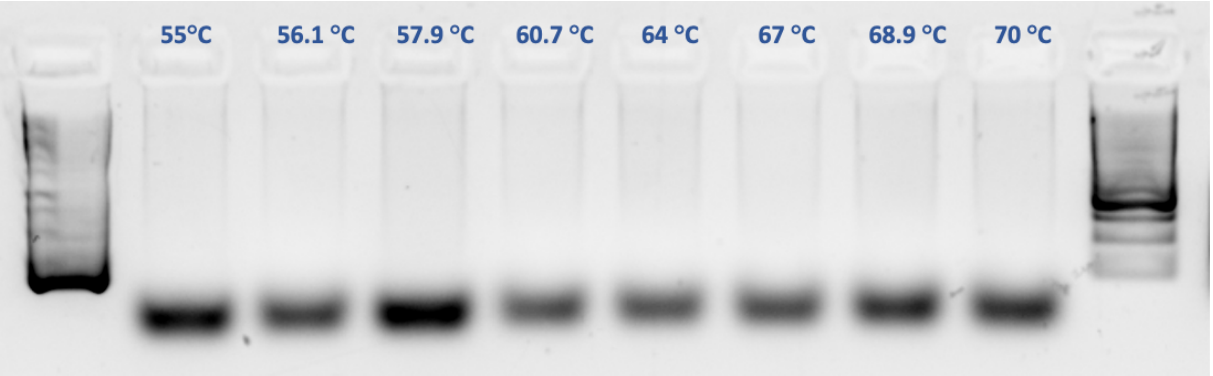

Figure 31. Example of PCR with VIN3 primers that did not yield any bands. Here, a gradient PCR spanning from 55 °C up to 70 °C starting from the second well. As seen, no bands are present, only residues from the samples applied on the gel.

3.7 CRISPR/Cas9 knock-out constructs

3.7.1 Designing gRNAs for CRISPR/Cas9 knock out

Four gRNAs were designed for each of *VRN1* and *VIN3*. Using CRISPOR (Concordet and Haeussler, 2018) gRNAs were chosen based on different parameters like specificity, off-targets and efficiency. The gRNAs were annotated in the gene sequences in Benchling [Biology Software]. (2022). For *VRN1* the gRNAs best suited were in the 5'UTR just before exon 1, as seen in Figure 32. The 2 first gRNAs were the ones included in the constructs.

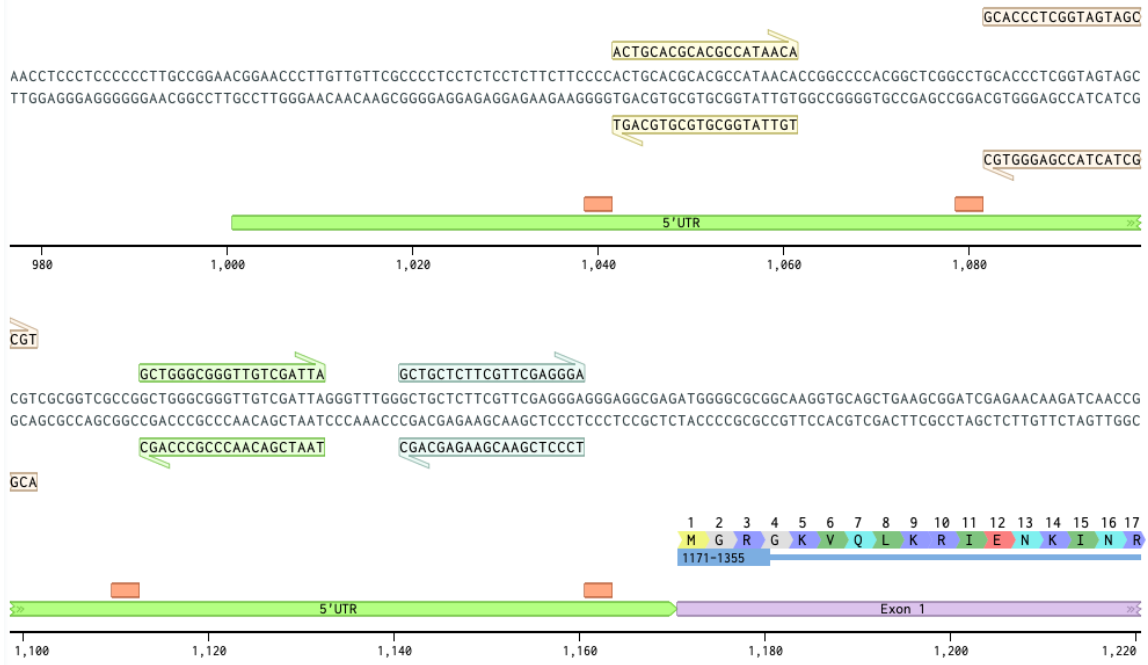

Figure 32. Overview of gRNA 1-4 in VRN1 as shown in Benchling. gRNA1 is seen in the beginning of the 5'UTR while gRNA 4 is closest to exon 1. PAMs are annotated in red.

As seen in Figure 33, all the gRNAs best suited for *VIN3* are in the first exon. Same as with the *VRN1* genes, the first 2 gRNAs in *VIN3* were also the ones included in the constructs.

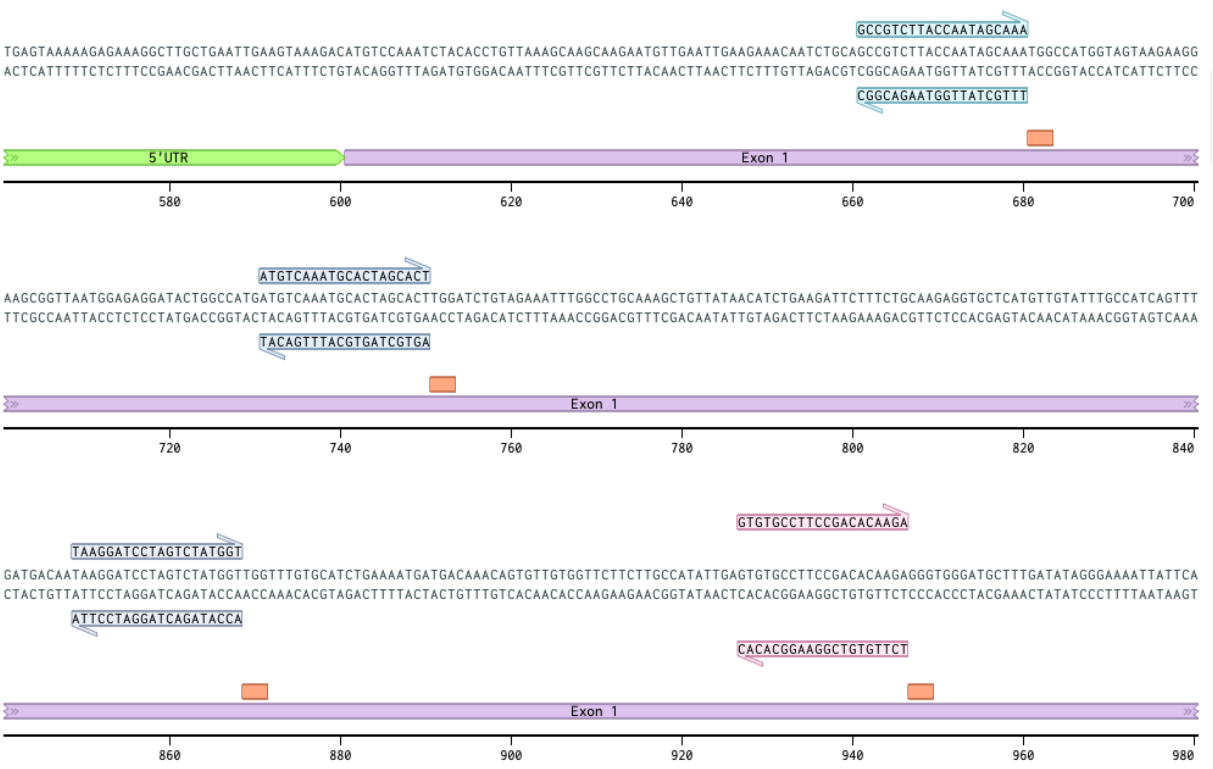

Figure 33. All 4 gRNAs for VIN3 in exon 1, annotated in Benchling. PAMs are annotated in red.

3.7.2 Designing and confirming gRNA primers for *VRN1* and *VIN3*

After designing and annotating gRNAs for both genes, the gRNA sequences were used to successfully design and amplify gRNA primers following the method from Grützner et al. (2021). Figure 34 shows the confirmation of 3 of the 4 primers designed for *VRN1*. By looking into the sequence of the pAGM9037 vector the expected size was 8407 bp while the expected band size for the primers was approximately 640 bp. As seen from Figure 34 the bands correspond appropriately to the expected values. Subsequently, when amplifying and validating the *VIN3* primers, band sizes were the same as those achieved with the *VRN1* primers.

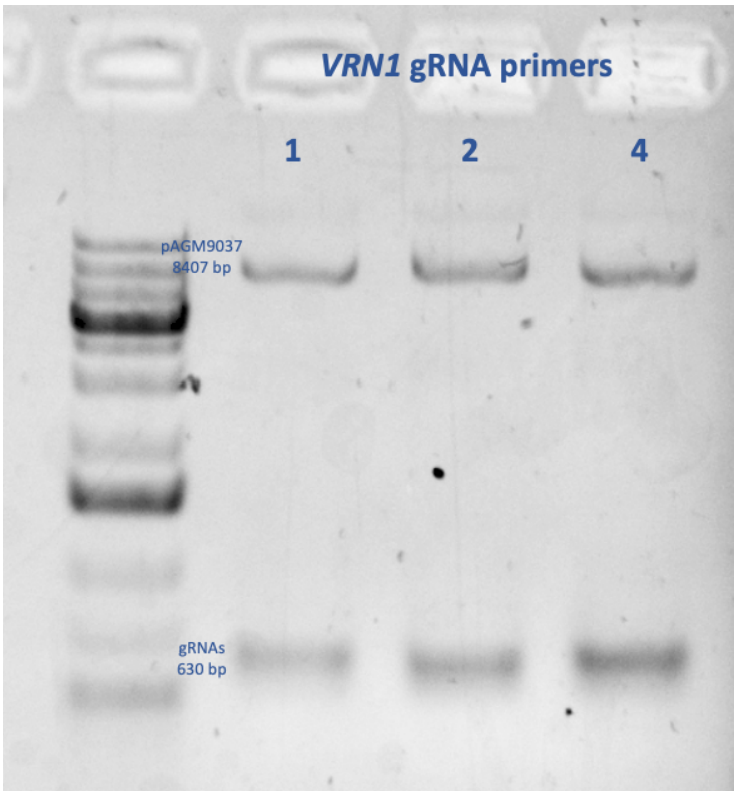

Figure 34 . Confirmation of VRN1 gRNA primers 1, 2 and 4 while waiting for gRNA3 to arrive. 8407 bp is the pAGM9037 vector, while the gRNAs have a band size of 630 bp.

3.7.3 Level 1 constructs

The restriction ligation was successful for both genes. Each gRNA was ligated into the corresponding level 1 pICH-plasmid together with the U6 promoter from the pAGM38869 plasmid and the pAGM9037 vector. White colonies grew on the blue/white selection plates, meaning the LacZ promoter was successfully removed from the pICH plasmids. Figure 35 and Figure 36 shows the successful restriction digestion with BpiI of the *VIN3* and *VRN1* gRNA primers, respectively, with the expected band sizes of the gRNAs at ~200 bp and the pICH plasmids at ~4000 bp.

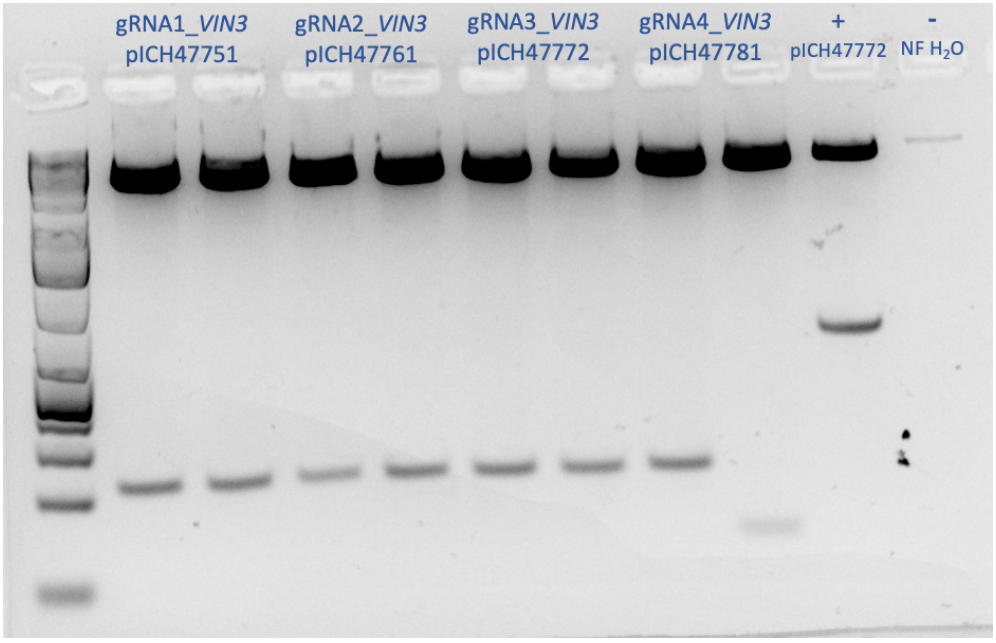

Figure 35. Restriction digestion BpiI with level 1 VIN3_gRNA_pICH plasmids in 2% gel with pICH47772 as a positive control and NF H2O as a negative control. pICH-plasmids have a ~4000 bp band size and gRNAs have a ~200 bp band size.

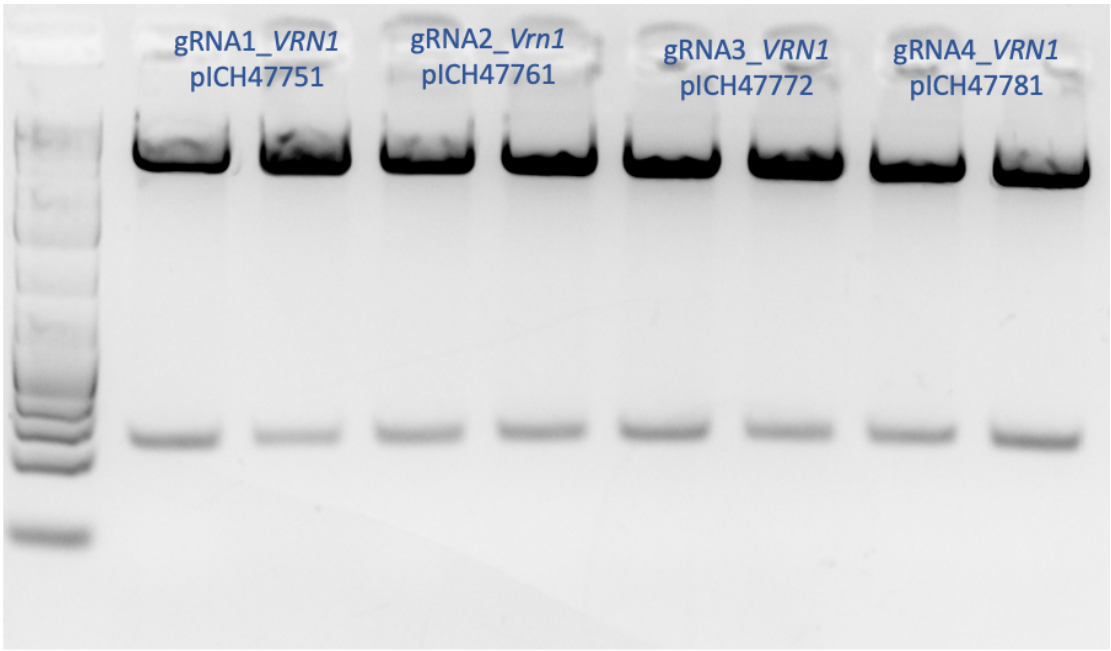

Figure 36. Restriction digestion BpiI with level 1 VIN3_gRNA_pICH plasmids in 2% gel. pICH-plasmids have a ~4000 bp band size and gRNAs a ~200 bp band size.

3.7.4 Level 2 constructs

Level 2 constructs for both *VIN3* and *VRN1* were made *in silico* using the NEBridge Golden Gate Assembly tool to illustrate the position of the two gRNAs in the pAGM65905 vector, as

well as performing the restriction digestion with ApaL1 to show the expected number of bands as well as their sizes. The results of this are depicted in Figure 37 and Figure 38 where 3 cut sites can be seen when using ApaL1 and two gRNAs are arranged in succession.

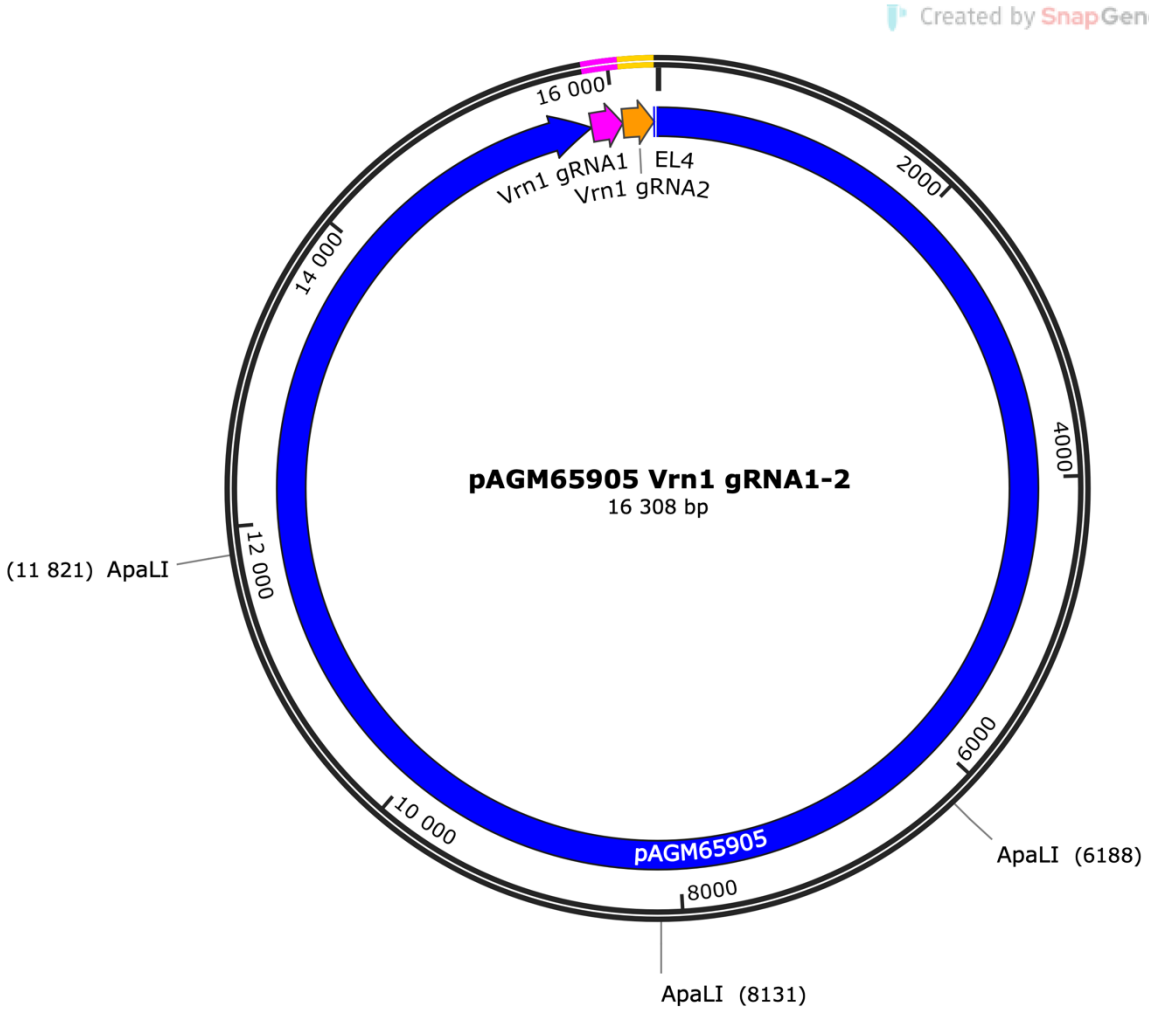

Figure 37. In silico representation of the level 2 VRN1 construct with gRNA1 and gRNA2 added to the pAGM65905 vector. ApaL1 restriction digestion gives 3 bands for a successfully created level 2 construct. Illustration created with SnapGene.

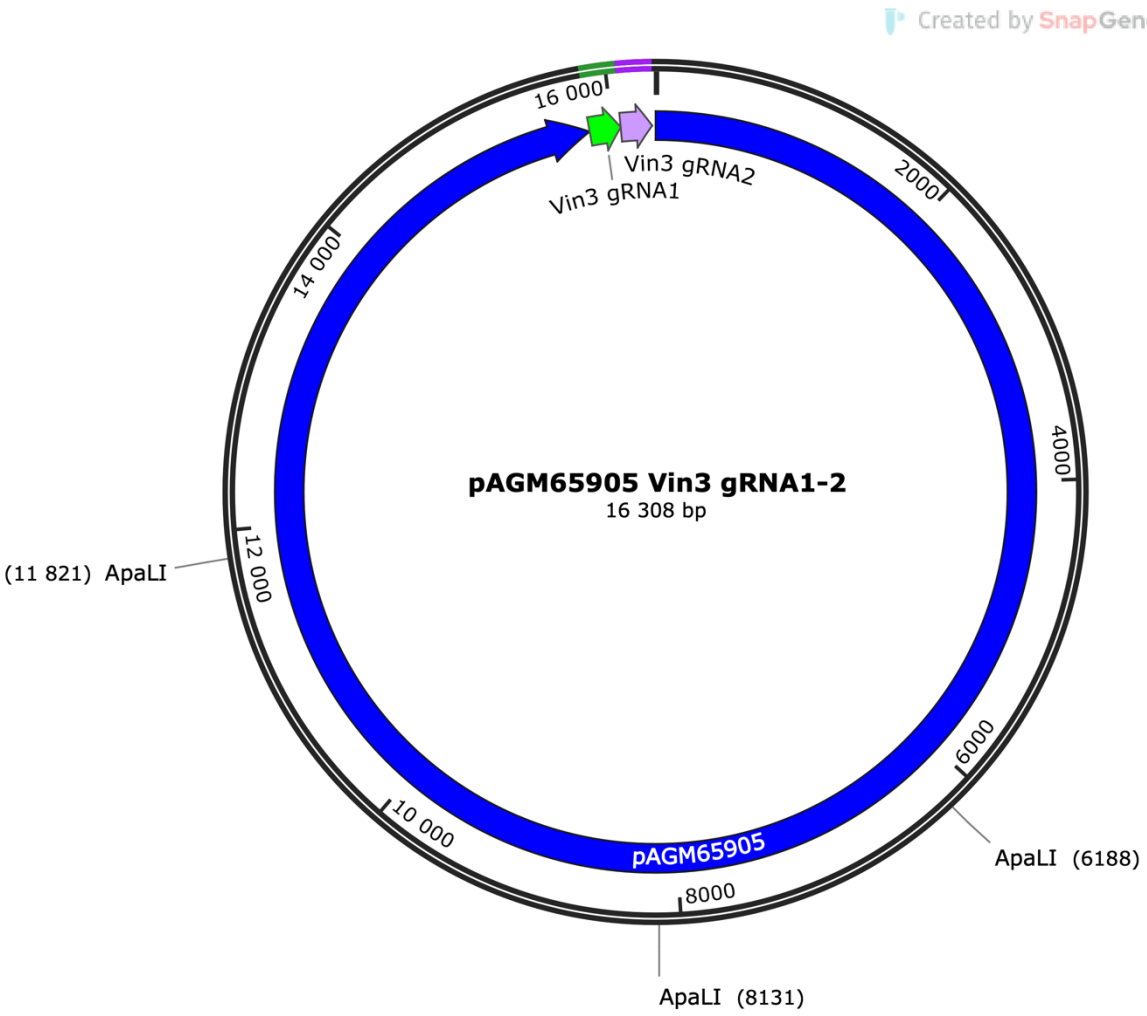

Figure 38. In silico representation of the level 2 VIN3 construct with gRNA1 and gRNA2 added to the pAGM65905 vector. ApaL1 restriction digestion gives 3 bands for a successfully created level 2 construct.

As seen from the *in silico* results, three bands were expected when using ApaL1 for the restriction digestion of the level 2 constructs. Figure 39 shows the results of the actual ApaL1 restriction digestion confirming the level 2 constructs. An empty vector was expected to have 4 fragments at 6321 bp, 4483 bp, 3690 bp and 1943 bp, while as previously stated a positive level 2 constructs with *VRN1* and *VIN3* generated with ApaL1 restriction digestion would generate 3 fragments. These band sizes would be 10675 bp, 3690 bp and 1943 bp. Eleven white colonies were collected, and all samples were run to confirm the constructs.

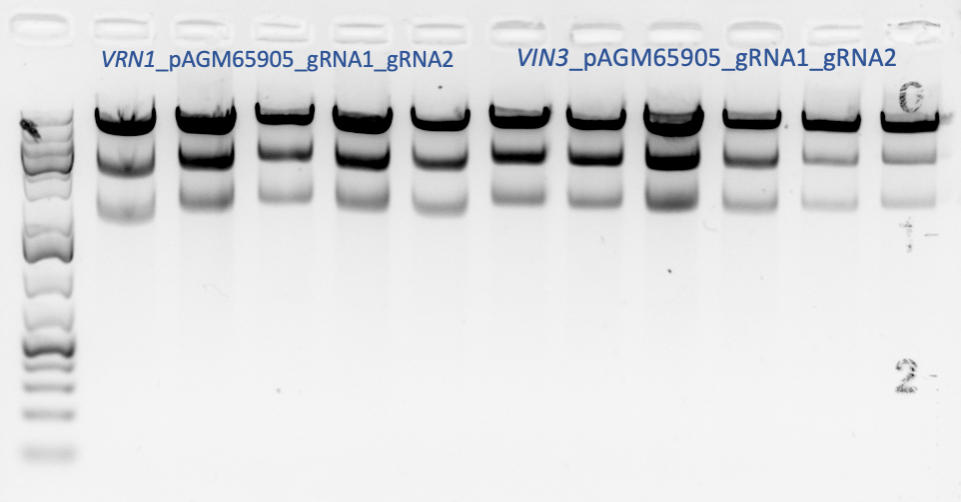

Figure 39. Gel picture of level 2 construct samples for VRN1 and VIN3 with 2 gRNAs, after restriction digestion with ApaL1. The gel show the expected three bands, as seen from in silico restriction digestion. Six samples of VRN1 and five samples of VIN3 were tested.

3.7.5 Transformation of calli with level 2 constructs

The subcultured calli transformed with the level 2 *VRN1* construct showed no sign of extensive *Agrobacterium* overgrowth and showed some development of new tissue, meaning that the transformation was successful and that the calli can be further maintained and developed for regeneration. The *VIN3*-transformed calli was also producing new tissue on the selection medium, but was discarded due to fungal contamination.

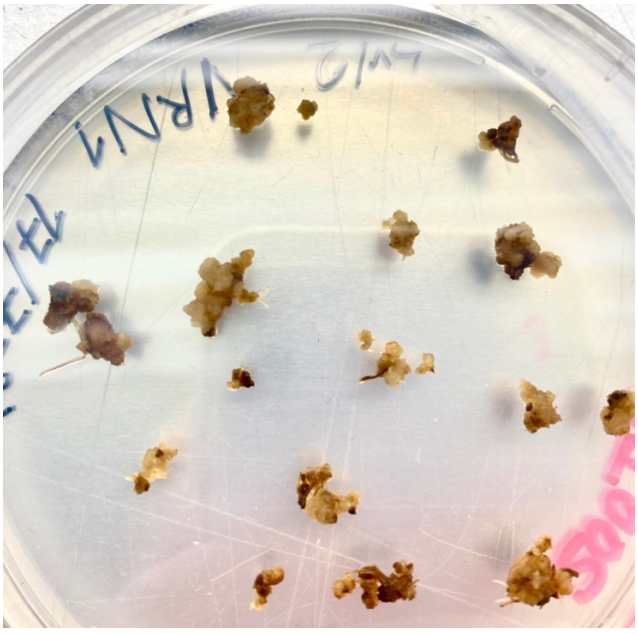

Figure 40. Agrobacterium-transformed perennial ryegrass calli with the level 2 VRN1 construct containing two gRNAs. Lighter areas on the calli show that the calli is developing and producing new tissue.

4. Discussion

4.1 Role of vernalization genes in flowering

The ability to synchronize the time of flowering with the surrounding environmental conditions through vernalization is important for perennial ryegrass since it secures reproductive success as well as contributing to seed production and fertilization.

In this study, the Fagerlin-201 and Fagerlin-204 genotypes of the "Fagerlin" cultivar, differ in their vernalization requirements. Fagerlin-201 does not require vernalization, while Fagerlin-204 needs vernalization to flower. This means that the expression of the important genes *VRN1* and *VIN3* differs, when the plants are exposed to shorter days in cold periods (Islam, 2015).

During the growing season of forage grasses, it is common to perform three cuttings. For some forage crop species, like the timothy cultivar "Grindstad", flowering starts early in the spring which contributes to an increased yield by the second and third cut (Höglind et al., 2005). However, for perennial ryegrass, the flowering is delayed, partially due to the vernalization requirement and the expression of *VIN3*. In the third cut, the yield tends to decrease (Laidlaw, 2005). This could be avoided by utilizing crispr-cas9 and transformation protocol established in this thesis. By promoting early flowering of perennial ryegrass, the yield could improve by the second and third cuts of the season. This can be done by knocking out *VIN3* and overexpressing *VNR1*. This will remove *VIN3*s repression of *VRN2* and therefore also alleviate *VRN1*, which in terms would express *VRN3* and promote flowering. By also overexpressing *VRN1,* early flowering would be ensured, since it would lead to the expression of *VRN3* and promotion of flowering to a greater extent.

On the other hand, in areas where the winters are colder and spring comes later, for example in northern parts of Norway, delayed flowering would be of interest to help the plants to survive the cold temperatures better, then overexpression of *VIN3 w*ould be a good option. In Fagerlin-204 the vernalization requirement is present, and as seen from the results provided by Islam (2015) expression of *VIN3* is high during cold and short days. By overexpressing this gene through Gateway cloning, this expression could increase

substantially. This would ensure the repression of *VRN2*, and therefore *VRN1* and *VRN3*, causing a delayed flowering. By having plant tissue with overexpressed *VIN3*, the *VIN3* mRNA would most likely stay at a higher level for a longer time, even when temperatures start to slightly increase.

This thesis mainly focused on producing calli from "Fagerlin" plant material and designing constructs for overexpression and knock-out for these two important genes, *VRN1* and *VIN3*. Hence, studying these genes and observing what would happen when knocking them out in the "Fagerlin" plant material is of interest, especially regarding the economic importance of perennial ryegrass.

4.2 Designing gRNAs and constructs

Originally, 4 gRNAs were designed for *VRN1* and *VIN3*. gRNA design is done in order to be able to target the specific region of interest within the gene. After retrieving the gene sequences, the CRISPOR program (Concordet and Haeussler, 2018) was utilized. This software was utilized based on recommendations from collaborators within this project, as well as the platform having one of the recent *Lolium perenne* genomes in their selection of databases.

The selection of an appropriate target site and the design of an effective gRNA towards this target is what largely determines the success of a CRISPR project (Hassan et al., 2021). The CRISPOR programme provides scores for each gRNA that is generated with parameters like specificity, efficiency, and off-targets (Concordet and Haeussler, 2018). The gRNAs position in the sequences is also given. This is of importance since the site in which the gRNA is located can affect the efficiency of the editing purpose (Hassan et al., 2021). A gRNA located in the first exon is likely to be more successful for a knock-out since the reading frame will be disturbed at an earlier point in the sequence, rather than at the very end, where it might not have the same effect since most of the gene would already be transcribed (Hus et al., 2020, Hassan et al., 2021). For this project, the goal is to knock out the *VRN1* and *VIN3* genes using CRISPR/Cas9, and therefore all gRNAs were either located in the first exon or the 5'-UTR to increase efficiency.

4.3 Overexpression

The construction of overexpression constructs using Gateway cloning was successful for 1 of the 2 genes focused on in this project, *VRN1*. For this gene all steps yielded positive results, leading to the transformations of the calli, which also provided positive results and signs of a successful transformation.

Although the same method was utilized for both genes, somehow, the *VIN3* primers did not show any bands on the gel, even when using different types of DNA and redesigning the primers. There could be several explanations for why this was the case, given that this is a complex and intricate procedure, with several steps, all of which could be prone to errors. For one, there could be faults linked to the gene sequence. The sequence could have been incorrectly annotated, leading to the primer sequences being retrieved from a part of the sequence outside of the coding regions. The introduction of the attB sites could also change the function or expression of the gene (Reece-Hoyes and Walhout, 2018). Another reason for the failed construction of *VIN3* could be due to the material utilized, although the same material was used for *VRN1*, as well as both cDNA and gDNA from both Fagerlin-201 and Fagerlin-204 were utilized in an attempt to produce positive results. The annealing temperature is also not likely to be the cause, since temperatures spanning from 43 °C up to 71 °C were tested.

Lv et al. (2014) have also made overexpression constructs added to calli, for *Brachypodium* and wheat, to study the effect of increased *FT1* expression on floral development. However, Gateway cloning was not utilized for overexpression, but rather binary vectors containing the ubiquitous 35S promoter together with a maize ubiquitin Ubi promoter (Lv et al., 2014). It has been reported that 35S promoters have weaker effects in monocot species, such as rice, and that for rice plants either Gateway- or only the maize ubiquitin promoters are more suitable for transgenic experiments (Tsuda et al., 2022). For this project, two destination vectors were used, in order to ensure the overexpression of the gene. The pMDC32 contains a 35S promoter and no reporter (Curtis and Grossniklaus, 2003), while the pANIC6A contains a maize ubiquitin promoter and a red fluorescent promoter (RFP) (Mann et al., 2012). This means that the pMDC32 vector only makes a complete coding sequence for the gene, while the pANIC6A will produce a fusion with the RFP, making it easier to confirm the

transformation. As stated earlier the Gateway cloning system contains vectors functioning as ubiquitous promoters, targeting all parts of the organism containing the targeted gene sequence for overexpression (Akbari et al., 2009). This could imply that the use of Gateway cloning for this experiment was advantageous compared to the other methods described here, due to its efficiency (Karimi et al., 2007).

4.4 Calli production

The calli produced from the "Fagerlin" seedlings were of good quality and were utilized for calli transformations with both the overexpression and the CRISPR/Cas9 knockout constructs. The protocol provided by Bajaj et al. (2006) was modified for this project. The seedlings were cut into regions, instead of being cut longitudinally. However, this did not seem to affect the regeneration of calli.

Generally, all plants are equipped with the trait of totipotency. This is a plant's ability to generate a whole new plant through somatic embryogenesis from any single plant cell, without any need for fertilization (!!! INVALID CITATION !!! (Fehér, 2019, Su et al., 2021)). In terms of tissue culture, somatic calli can be produced from any type of plant tissue, such as leaves, stems etc placed on nutrient media to produce somatic tissue. The important plant hormones, auxins and cytokinins are key regulators for somatic embryogenesis and need to be added to the media to induce the process.

The production of meristematic calli from meristems retrieved from the Fagerlin-201 and Fagerlin-204 plants did not yield positive results as with the "Fagerlin" seed protocol. Here, a protocol established by collaborators at TalTech University was utilized, where it had been proving positive results. However, for this project, the results were not as adequate. There could be several reasons causing these results, one being simply collecting the wrong part of the plant shoot or meristem, or any of the solutions used for the washing procedure being too strong for the meristems, such as the bleaching solution. Furthermore, the plant material itself could also be a contributing factor. The particular genotypes could perhaps have less totipotency as this generally can depend on the genotype and plant material (Delporte et al., 2012, Schrauf et al., 2022). Generally, when plant material is placed on

media, the expected outcome would be the formation of calli, if not meristematic tissue, at the very least somatic tissue, however, some genotypes may lack or display less of this ability, as plant transformation methods generally can be highly genotype-dependent (Maren et al., 2022).

The generation of embryonic calli did not yield successful results. An important factor here would be the timing at which the seeds were collected, resulting in immature seeds without any mature embryos for calli production. The seeds were collected in late summer/early fall, meaning that the plants would have already produced seeds at an earlier time. Retrieving seeds from the plants in the field at an earlier time would likely have provided positive results, with mature seeds. Another factor might be the anatomy of mature monocotyledonous embryos, like wheat, maize and perennial ryegrass, where the SAM is enclosed and surrounded by the coleoptile and several layers of leaf primordia. This must be removed to gain physical access needed for DNA delivery technologies (Ye et al., 2023). On the other hand, immature embryos have been used for the transformation of wheat, however, it is stated that the quality of such immature embryos is the most important factor for efficient transformation (Ishida et al., 2015). For wheat, good embryos could only be obtained from vigorous plants grown in greenhouses that are well-conditioned in terms of temperature and light conditions (Ishida et al., 2015). One could expect that the same could be possible for immature perennial ryegrass seeds,

For this project, perennial ryegrass leaves were surface sterilized and put on MS media containing the synthetic auxin 2,4-D in the dark for 6 weeks to produce somatic calli. Leaf tissue produced by seedlings was also placed on media to make calli. However, no calli were produced, even when keeping the plates for longer than 6 weeks, and even without any contamination. Normally, one would expect such plant material to yield somatic calli (Delporte et al., 2012, Chib et al., 2020).

McSteen (2010) suggests that monocots and grasses in particular respond differently to synthetic or exogenous auxin compared to dicots, caused either by differences in translocation or quick degradation of exogenous auxin. Auxin is a key regulator for somatic embryogenesis to produce somatic calli (Su et al., 2021). This, in addition to the typically

thick cuticle of monocot grasses, could explain the absence of somatic calli from the leaf tissue of perennial ryegrass.

4.5 CRISPR/Cas9 knockout

In this thesis, the CRISPR/Cas9 method was utilized to make constructs that could knock out the *VRN1* and *VIN3* genes in perennial ryegrass calli. Usually, when obtaining site-specific mutations in plants, they are generally transformed using the gRNA expression cassette together with the Cas9 gene in a construct. The approach for this project is based on the technique described by Grützner et al. (2021). Here the focus lies on the efficiency of the CRISPR/Cas9 constructs, and it is described that the features of the constructs, as well as the nature and source of the promoters and terminators, can largely affect the efficiency of introducing the mutations in the genes. Grützner et al. (2021) worked with *Arabidopsis thaliana* for optimization of the Cas9 construct efficiency by investigating codon usage, the presence, and absence of introns, as well as the number of nuclear localization signals (NLSs). The presence of introns has been proven to be beneficial for gene expression, one of the reasons may be that plant genes generally contain several introns. Adding constructs rich with introns may lead to a more successful transformation since the tissue will be more acceptant of the intronized construct (Grützner et al., 2021).

To assemble the Cas9 constructs specific empty plasmids from the MoClo kit (Weber et al., 2011) together with the more specific intron-optimized SpCas9 plasmids from the zCas9i kit (Grützner et al., 2021) are utilized to produce level 1 and level 2 constructs. Level 2 constructs consist of a specific zCas9i vector where level 1 constructs specific for each gRNA together with an end-linker plasmid are ligated and replace the Lac-Z promoter, enabling blue-white selection.

Originally, 4 gRNAs were designed for the knockout of the *VRN1* and *VIN3* genes. However, after several tries of constructing the level 2 constructs with all 4 gRNAs with the corresponding plasmids and end-linker, all 4 gRNAs were never present when reviewing the sequencing results of the constructs created. Therefore, due to time limitations, only 2 gRNAs were added to the level 2 constructs, since this had proven successful for other members in the laboratory. When confirming and sequencing these constructs, both gRNAs

were present and in the right order for both genes, as seen from the *in silico* results of the restriction ligation with ApaL1. Regarding the time frame for this thesis, no extensive trouble-shooting was performed regarding this issue, except testing different concentrations and samples of the different constructs and plasmids. However, one possible theory could be off-target effects, the specificity or efficiency of each of the gRNAs (Zhang et al., 2014), although this was accounted for when choosing grNAs with the CRISPOR program (Concordet and Haeussler, 2018).

There are also other ways of utilizing CRISPR/Cas9 besides the zCas9i constructs from Grützner et al. (2021), using other types of binary vectors without designing the same type of constructs. Okada et al. (2019) utilized CRISPR/Cas9 to produce knock-out lines of wheat where the first generation exhibits male sterility. Here tDNA of a binary vector from *Agrobacterium* was used for transformation, where the goal was to demonstrate CRISPR/Cas9 and to knock out the *Ms1* gene, which yielded positive results (Okada et al., 2019). Kishchenko et al. (2020) gives an overview of how CRISPR/Cas9 knockout can be used to modulate seed dormancy and flowering time in major crops like maize, rice and soybean. Here it is described that the knock-out of a key photo response regulator in maize using CRISPR/Cas9 caused early flowering during long days. Here pCambia and pGreen binary vectors were utilized (Xing et al., 2014).

It is described that the CRISPR/Cas9 provides a straightforward and rapid method for gene editing in forage grasses and that this method is predicted to be used routinely for such an economically important forage crop like perennial ryegrass (Zhang et al., 2020).

4.6 Conclusions and future perspective

This project focused on the vernalization genes *VIN3* and *VRN1* in the Fagerlin cultivar of perennial ryegrass. Both constructs for overexpression and CRISPR/Cas9 knockout were designed, by using the gene sequences to create primers and gRNAs. Calli was also produced from Fagerlin seeds, which were later transformed with the constructs using *Agrobacterium* mediated transformation techniques. Within the time frame of this thesis, there was no time to confirm the transformed calli or waiting for them to regenerate. However, results were seen in the calli, since new cells were developed in the tissues.

For this study, there is additional work that can be done for validation and further confirming the results found in this study. The already transformed calli in this thesis, could be further maintained to allow regeneration and the production of transgenic plants. These plants can be utilized for phenotypical validation of the *VRN1* and *VIN3* gene knockouts. There could also be done additional work on Fagerlin-201 and Fagerlin-204 plant material. Here the successful results were provided by the "Fagerlin" plant material which is the origin of both genotypes. Further, studies could focus on producing calli specifically from both Fagerlin-201and Fagerlin-204 with the successful method from Bajaj et al. (2006) and utilize the constructs for this plant material. These plants could further be tested in environments mimicking both short days and long days, to study any possible changes in vernalization and hence flowering.

Establishing the protocols needed to produce a CRISPR/Cas9 construct in perennial ryegrass illustrates what a time-consuming process it can be, with numerous points existing where potential complications or errors can arise. However, with an established and complete protocol for constructing CRISPR/Cas9 knockout constructs in perennial ryegrass, shown in Figure 13, there are several opportunities.

CRISPR/Cas9 systems can be used for improving crop plants and studying their gene functions, and such an efficient system is needed in perennial ryegrass (Kumar et al., 2022). This is due to the economic importance of perennial ryegrass as well as its position in Norway and other countries as an important and nutritious forage grass and crop. An effective CRISPR/Cas9 knockout system for perennial ryegrass can accelerate the rate of plant breeding as well as gene function studies in this plant. This could lead to the improvement of important crop traits, such as resistance to disease and pests, yield enhancement and like the focus for this study, stress tolerance like frost and vernalization requirements.

Utilizing CRISPR/Cas Cas9 to target specific genes that are of interest by knocking them out, insights on different development and biochemical pathways could be understood. Further understanding of the interactions between different genes under different environmental

conditions are also of interest. Moreover, by establishing a knockout CRISPR/Cas Cas9 protocol in perennial ryegrass, one could utilize it as a model system for research in related species of monocot grasses that also are of economic importance.

5. Supplementary materials

Supplementary table 1. List of primers used for overexpression and CRISPR/Cas9 constructs

Primer name	Primer sequence (5' to 3')
***VRN1*_attB_F**	AAAAAGCAGGCTTAATGGGGCGCGGCAAGGTGCAG
***VRN1*_attB_R**	AAGAAAGCTGGGTCGCCATTGTTGATGTGACTCAC
***VIN3*_attB_F**	AAAAAGCAGGCTTAATGTCCAAATCTACACCTGTT
***VIN3*_attB_R**	AAGAAAGCTGGGTCTCTTTTGTCCAAATGGCATAG
attB1_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT
attB1_R	GGGGACCACTTTGTACAAGAAAGCTGGGTC
pDONR207_F	TCGCGTTAACGCTAGCATGGATCTC
pDONR207_R	GTAACATCAGAGATTTTGAGACAC
gRNA_VIN3_1_critarx	TTGGTCTCAATTGTGTGCCTTCCGACACAAGAGTTTAGAGCTAGAAATAG
gRNA_VIN3_2_critarx	TTGGTCTCAATTGTAAGGATCCTAGTCTATGGTGTTTAGAGCTAGAAATAG
gRNA_VIN3_3_critarx	TTGGTCTCAATTGCCGTCTTACCAATAGCAAAGTTTAGAGCTAGAAATAG
gRNA_VIN3_4_critarx	TTGGTCTCAATTGATGTCAAATGCACTAGCACTGTTTAGAGCTAGAAATAG
gRNA_VRN1_1_critarx	TTGGTCTCAATTGACTGCACGCACGCCATAACAGTTTAGAGCTAGAAATAG
gRNA_VRN1_2_critarx	TTGGTCTCAATTGCACCCTCGGTAGTAGCCGTGTTTAGAGCTAGAAATAG
gRNA_VRN1_3_critarx	TTGGTCTCAATTGCTGGGCGGGTTGTCGATTAGTTTAGAGCTAGAAATAG
gRNA_VRN1_4_critarx	TTGGTCTCAATTGCTGCTCTTCGTTCGAGGGAGTTTAGAGCTAGAAATAG
critarev	ATGTACGGCCAGCAACGTCG
l1f primer	GTGGTGTAAACAAATTGACGC
Level 2 seq. primer	TGTGGTTGGCATGCACATAC

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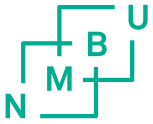

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