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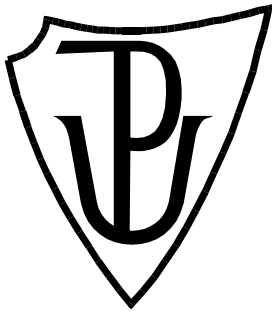

Nemendelistická dědičnost u rostlinných hybridů

DIPLOMOVÁ PRÁCE

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Studijní program:	N0512A130007 Biotechnologie a genové inženýrství
Studijní obor:	Biotechnologie a genové inženýrství
Forma studia:	Prezenční
Vedoucí práce:	**Mgr. Jana Szecówka Ph.D.**
Rok:	2024

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V Olomouci dne ..... ……..

Podpis studenta

Acknowledgment

I would like to thank my supervisor Mgr. Jana Szecówka Ph.D. for professional guidance, time, support and patience during my thesis. I would also like to thank the group leader RNDr. David Kopecký Ph.D. for the opportunity to work on my thesis in the wide hybridization group and for the provided background and resources. Of course, I would also like to thank the other members of the group as well as other employees of the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic.

Bibliografická identifikace

Jméno a příjmení autora	Bc. David Tatalák
Název práce	Nemendelistická dědičnost u rostlinných hybridů
Typ práce	Diplomová
Pracoviště	Ústav experimentální botaniky AV ČR
Vedoucí práce	Mgr. Jana Szecówka Ph.D.
Rok obhajoby práce	2024

Abstrakt

Genomová dominance je fenomén, při kterém dochází kporušení pravidel mendelistické dědičnosti. Poměrně častý je u mezidruhových a mezirodových hybridů, tedy jedinců vzniklých křížením dvou různých druhů či rodů. U hybridů se jeden subgenom může stát dominantním, zatímco submisivní subgenom může být eliminován. Genomová dominance byla důkladně studována u hybridů *Festuca x Lolium*. U tohoto hybrida je dominantní subgenom *Lolia*, tato skutečnost se projevuje na několika úrovních od celého genomu, přes chromozomální úroveň až na úrovneň jednotlivých genů. Bylo prokázábo, že jsou *Festukové* chromozomy v průběhu generací eliminovány, ale podrobné znalosti o mechanismech stále chybí. Bylo nastíněno, že umlčení variant *Festukových* kinetochorových genů může hrát roli v celém procesu.

Prvním cílem bylo vytvoření knock-out linií *A. thaliana* pro kinetochorové geny *CENPC* a *NDC80*, které měli být následně využity pro další výzkum. Byla použita technika CRISPR/Cas9. V případě genu *CENPC* nebylo žádné z pozitivně transformovaných semen životaschopné, což lze přičíst důležitosti genu *CENPC* v rostlinách. Pro gen *NDC80* jsme získali celkem 17 linií s mutací zavedenou v zájmovém genu v T1 generaci, ze které jsme propagovali nejslibnější linie do T2 generace. Ty budou použity k vytvoření mezidruhových hybridů k napodobení homologního specifického genového umlčování pozorovaného u *Festuca x Lolium* hybridů.

Druhým úkolem této práce bylo otestovat, zda mohou být homoeologní kinetochorové proteiny volně zaměňovány během sestavování kinetochorů. Pomocí metody Yeast two-hybrid jsme chtěli analyzovat vazbu mezi interagujícími partnery kinetochorového komplexu MIS12 a NNF1 a NDC80 a NUF2 a odhalit změny v interakcích při kombinaci homologních nebo homoeologních proteinů. Vzhledem k přetrvávajícím problémům jsou však výsledky neprůkazné.

Klíčová slova	CRISPR/Cas9, *Festulolium*, Genomová dominance, Kinetochorové proteiny, Mezidruhová hybridizace, Nemendelistická segregace chromozomů, Nemendelistická dědičnost, Protein-proteinové interakce, Rostliny, Yeast two-hybrid

Počet stran	85
Počet příloh	15
Jazyk	Anglický

Bibliographical identification

Author's first name and surname	Bc. David Tatalák
Title	Non-mendelian inheritance in plant hybrids
Type of thesis	Master
Department	Institute of Experimental Botany of the Czech Academy of Science
Supervisor	Mgr. Jana Szecówka Ph.D
The year of presentation	2024

Abstract

Genome dominance is a phenomenon in which the rules of Mendelian inheritance are violated. It is relatively common in interspecific and intergeneric hybrids, i.e. individuals created by crossing two different species or genera. Within the hybrids, one subgenome may become dominant while the submissive subgenome may get eliminated. Genome dominance has been well studied in *Festuca x Lolium*. The *Lolium* subgenome is dominant with this hybrid which is manifested at several levels including whole genome, gene as well as chromosomal level. It has been reported that *Festuca* chromosomes get eliminated over the generations but detailed knowledge of the mechanisms is still lacking. It has been proposed that silencing of *Festuca* variants of kinetochore genes plays a role in this process.

The first goal was the establishment of knock out lines of *A. thaliana* for the kinetochore genes *CENPC* and *NDC80* to be available for further research. The CRISPR/Cas9 technique was used. In the case of CENPC gene, none of the positively transformed seed was viable, which could be attributed to the importance of CENPC gene in plants. For the NDC80 gene, we obtained a total of 17 lines with the mutation introduced within the gene of interest in T1 generation from which we propagated the most promising lines into T2 generation. These will be used to create interspecific hybrids to mimic the homoeologous specific gene silencing observed in *Festuca x Lolium.*

The second task of this work was to test whether the homoeologous kinetochore proteins can be freely interchanged during the kinetochore assembly. Using yeast two-hybrid assay, we wanted to analyse the binding between interacting partners of the kinetochore complex MIS12 and NNF1 and NDC80 and NUF2 and estimate changes in the interactions when homologous or homoeologous proteins are combined. However, due to persistent problems, the results are inconclusive.

Keywords	CRISPR/Cas9, *Festulolium*, Genome dominance, Interspecific hybridization, Kinetochore protein, Non-mendelian chromosome segregation, Non-mendelian inheritance, Plants, Protein-protein interaction, Yeast two-hybrid
Number of pages	85
Number of appendices	15
Language	English

Content

Objectives of master's thesis

- Using the Yeast two-hybrid method, demonstrate how individual kinetochore proteins interact with each other in *Festuca* and *Lolium* plants.
- Prepare and characterize *Arabidopsis thaliana* mutant lines for future research.
- Literature review on the topic of plant interspecific hybridization, focusing on the kinetochore proteins and their role during so-called genome dominance.
- Master the methods of molecular biology such as Gateway cloning, Yeast two-hybrid, Crispr/Cas9, and *Agrobacterium-mediated* transformation using the floral dip method.

1 Introduction

Interspecific hybridization is considered one of the driving forces of evolution in the plant kingdom. In this phenomenon, at least two organisms of a different species, and in some cases even of a different genera, cross. The resulting hybrids then carry at least two sets of genomes of different origins. The fact that there has been an increase in the amount of chromatin inside the cell nucleus, the cells have to adapt rapidly. Inside cells, therefore, major changes occur at all levels, from changes at the level of gene expression to changes in the very structure of the cell. It is not surprising that the hybridization process carries with it several advantages, but it is also associated with a number of difficulties (Rodionov *et al*., 2019; Kopecký *et al*., 2022).

One of the phenomena occurring in interspecific hybrids is genomic dominance. During genome dominance, genomes of different origins compete within hybrids. The result of this process is the dominance of one of the genomes over the other, while the second submissive genome is gradually eliminated to a certain extent, in some cases the complete elimination of the submissive genome may occur (Glombik *et al*., 2020; Glombik *et al*., 2021). Elimination occurs mainly during cell division when certain parts of the dominant genome are favored over the submissive. This leads to a violation of classical Mendelian inheritance (Majka *et al*., 2023)

A plant species regularly used to study genome dominance is the *Festulolium* species. *Festulolium* is a relatively newly created interspecific hybrid organism, which was created by crossing two relatively close species *Festuca* and *Lolium*. Genome dominance in hybrids is manifested by the dominance of the *Lolium* genome over the *Festuca* genome, and parts of the submissive *Festuca* genome are eliminated. However, the mechanisms behind this phenomenon are not well-known and studied (Kopecký *et al*., 2017; Glombik *et al*., 2021). Previous work by Majka *et al*. (2023), however, pointed out interesting facts regarding some kinetochore proteins, which could be related to the role of these proteins during genome dominance in the *Festulolium* species and potentially contribute to a greater understanding of the issue in the respective species and beyond.

2 Literary review

2.1 Polyploidy and interspecific hybridization

2.1.1 Polyploidy

The process of polyploidization is a phenomenon in which new individuals carrying more than two sets of chromosomes arise (Ranney, 2006). This phenomenon is very rare in the animal kingdom, exceptions can be found in some representatives of insects (Li *et al*., 2018), fish (Zhou and Gui, 2017), and amphibians (Schmid *et al*., 2015). In the case of plants, however, it is an important driving force in the evolution of most plant species. It has been shown that a large proportion of plant species existing today have undergone at least one process of polyploidization during their evolution (Soltis *et al*., 2015).

Among polyploids, we distinguish two main categories: autopolyploids and allopolyploids. Individuals with all sets of chromosomes originating from the same species are considered autopolyploids (Barker *et al*., 2016). The most important representatives include some species from the Brassicaceae family (Albertin *et al*., 2005), potatoes (De Haan and Rodriguez, 2016), and others. Autopolyploids can be further divided based on various genetic characteristics, such as chromosomal profile and behavior (Soltis *et al*., 2010). In such a case, we can distinguish between polysomic polyploids and disomic polyploids (Osborn *et al*., 2003; Scott *et al*., 2023). Allopolyploids are individuals that carry sets of chromosomes from two or more different species (Barker *et al*., 2016). However, these species must be relativelly closely related, otherwise interbreeding could not occur at all. The reason is the mutual homology of chromosomes, whereby the greater the differences between chromosomes originating from different species, the greater the probability that homologous chromosome pairing errors will occur during cell division (Sun *et al*., 2017; Svačina *et al*., 2020). The most important allopolyploid species include wheat (Marcussen *et al*., 2014), cotton (Wendel and Cronn, 2003), banana (Heslop-Harrison and Schwarzacher, 2007), tall fescue (Humphreys *et al*., 1995), and others.

2.1.1.1 Induction of polyploidy

The formation of polyploids can occur spontaneously in nature via two major mechanisms. The first is referred to as somatic doubling. Changes, more specifically endomitosis or

endoreduplication, occur during mitosis in zygote cells or apical meristematic tissue. The result is mixoploid organisms, which in extreme cases can be completely polyploidy (Ramsey and Schemske, 1998). However, compared to the second mechanism, it is far less important. The second way is the formation of unreduced reproductive cells. Most plants have been shown to be capable of producing unreduced reproductive cells. The resulting unreduced gametes can fuse both with other unreduced gametes and with reduced gametes (Ramsey and Schemske, 1998; Sattler *et al*., 2016).

Nowadays, however, the artificial creation of polyploids is much more discussed, as polyploidy often entails an improvement in some phenotypic and other properties of the resulting individuals. (Scott *et al*., 2023). Several new species or varieties have been artificially created over the years (Stanys *et al*., 2007; Kitamura *et al*., 2009; Mansouri and Bagheri, 2017). The most common methods used to induce polyploidy are radiation (Britt, 1996), nitrous oxide (Kitamura *et al*., 2009), other chemical treatments, such as colchicine (Stanys *et al*., 2006; Mansouri and Bagheri, 2017), and exposure to temperature shock (D'Amato, 1997).

2.1.2 Interspecific hybridization

The term interspecific hybridization describes the process in which individuals of different species cross. Subsequently, emerging hybrids carry the genetic makeup of both parents. In case whole genome duplication occurs after interspecific hybridization, the allopolyploids mentioned above arise. Within eukaryotic organisms, interspecific hybridization is most common in the plant kingdom and is now considered one of the important driving forces of plant evolution. At the same time, interspecific hybridization played and still plays an irreplaceable role in the domestication of agriculturally important crops and their subsequent breeding. Due to the complexity of the entire process and the subsequent changes that occur in hybrid organisms, allopolyploids are associated with many positive and negative impacts. (Rodionov *et al*., 2019; Kopecký *et al.,* 2022).

2.1.3 Advantages of polyploidy

As already said, polyploids have a few advantages over diploid plants. Among the most studied benefits of polyploidy are gene redundancy, loss of self-incompatibility, gain of

asexual reproduction, and heterosis. In the case of gene redundancy, the hybrid plant benefits from the fact that it contains multiple sets of chromosomes. Dominant wild-type alleles provide a certain level of protection against deleterious recessive alleles and thus protect the organism from the consequences of mutations (Comai, 2005). The second phenomenon allows hybrids to bypass mechanisms preventing self-fertilization (Miller and Venable, 2000) or favor asexual reproduction which helps in cases of absence of suitable partners, but the molecular basis of these responses is unclear (Comai, 2005). Heterosis refers to the fact that hybrid plants often have better characteristics than the parent diploid plants. This fact is most used in plant breeding because hybrids may show a higher yield, grow faster and taller and can be more resistant to biotic and abiotic stresses, (Hochholdinger and Baldauf, 2018). At the same time, it has been proven in allopolyploids that the greater the genetic difference between the parental plants, the higher the level of heterosis in the resulting hybrid plant (Birchler *et al*., 2010).

2.1.4 Disadvantages of polyploidy

In addition to the advantages mentioned above, allopolyploids are also associated with several disadvantages and problems. The first is the fact that there is more chromatin inside each polyploid cell than in diploid cells. Logically, more chromatin means more cell volume (Melaragno *et al*., 1993). The allopolyploid cells had to adapt quickly to the increased cell volume, so there are major changes in the structure of the entire cell and individual cell organelles (Jasencakova *et al*., 2003; Corredor *et al*., 2005). Higher chromatin content is also associated with higher instability of gene expression, as a large number of epigenetic changes occur in the cell. The aforementioned instability can also cause errors during cell division. (Comai, 2005). All the disadvantages, especially those described below, can, to a certain extent, participate in changes in the inheritance of certain alleles and thus contribute to their non-Mendelian inheritance (Ranney, 2006).

2.1.4.1 Epigenetic instability

Changes in epigenetics appear to be associated with the increase and remodeling of chromatin in allopolyploid cells. Epigenetic changes can give rise to new adaptive mutations, which result in the manifestation of a new plant phenotype. As a result of the large changes inside the cell, epigenetic changes are very rapid. The most common are cytosine methylation at

specific sites, rapid silencing of ribosomal RNA and protein-coding genes, and reactivation of dormant transposable elements (Liu and Wendel, 2003).

2.1.4.2 Difficulties in meiosis

The formation of stable bivalents is necessary for the correct course of meiosis. In the case of allopolyploids, the cell contains multiple sets of chromosomes originating from parents of different species. Terminologically, chromosomes originating from the same parent are called homologues, while chromosomes originating from different parents are called homoeologues. In most cases, interspecific hybrids prevent pairing between homoeologous chromosomes during cell division, so only homologues pair up. This phenomenon is conditioned by either the difference in chromosome sequences or by the presence of mechanisms of control. These mechanisms are often not found in the new hybrids, but take some time to develop (Kopecký *et al*., 2022). One example of such a mechanism is controlled by the *Ph1 (Pairing homoeologous 1)* gene found in wheat (Knight *et al*., 2010).

However, not all allopolyploids have or use such a mechanism. Among the hybrids, we find several species in which, during cell division, free pairing of homoeologous chromosomes occurs (Kopecký *et al*., 2022). This brings with it the possibility of introgression of certain genes or entire parts of chromosomes from one species to another (Kopecký *et al*., 2010). At the same time, there is a large increase in the overall variability and genomic instability of the resulting offspring. (Kopecký *et al*., 2022). Examples can be hybrids between *Allium cepa* and *Allium roylei* (Kofoet et al., 1990) and a hybrid between *Festuca* and *Lolium* species, referred to as *Festulolium* (Kopecký *et al*., 2010). In both hybrid species, the phenomenon of Meiotic drive occurs, which leads to the non-Mendelian inheritance and potentially subgenome dominance (Henikoff *et al*., 2001; Pardo-Manuel De Villena and Sapienza 2001; Kopecký *et al*., 2022).

2.2 Non-mendelian inheritance

Gregor Mendel's laws of inheritance laid the foundation for classical genetics, outlining the predictable patterns of trait transmission through simple rules of dominance, segregation, and independent assortment (Kar and Sarkar, 2022; Mackay and Anholt, 2022; Zhang, 2023). However, as research progressed, scientists uncovered exceptions to these rules, leading to

the recognition of non-Mendelian inheritance patterns. Deviations of trait inheritance from classical Mendelian inheritance can be based on several principles. The first is based on various epigenetic changes of the chromatin molecule, the second on various interactions between individual genes, when a set of individual genes participates in the resulting phenotypic expression, or one gene regulates the expression of another. Another important factor is the influence of environmental conditions on the expression of individual genes, which may or may not occur (Wolf *et al*., 2022).

2.2.1 Gene-gene interactions

In this subsection, I want to summarize all types of non-Mendelian inheritance, which are caused by some kind of interaction between individual genes. The first type is the so-called incomplete dominance, and its most extreme form is the co-dominance. In the case of incomplete dominance, the recessive allele is not completely silenced by the dominant and can manifest itself to some extent in the resulting phenotype. In co-dominance, neither allele is dominant, and both are expressed to the same extent, and the resulting phenotype is a combination of both alleles (Wesmiller and Grayson, 2023).

In classical Mendelian inheritance, it is assumed that each gene has only two alleles. In practice, however, this is not entirely true, and some genes occur in several forms. These alleles have a clearly ordered hierarchy among themselves (Vasisth *et al*., 2023). Similarly, Mendelian inheritance only works in cases where a given character is controlled by only one gene. However, many traits are conditioned by several different genes, and without the presence of all the mentioned genes, the phenotype will not manifest itself. In this case, we are talking about polygenic traits (Bentsen, 2005).

Another type is genetic linkage. This phenomenon occurs when genes on a chromosome are located in close proximity. These genes are linked to each other and during meiosis they usually do not split during crossing over and are always passed on together (Pulst, 1999). The last example is the so-called epistasis. Epistasis occurs when multiple genes express the same phenotype. This can result in a deviation from the Mendelian phenotypic split ratio of individuals (Mackay, 2014).

2.2.2 Epigenetic inheritance

We can understand the terms epigenetics or epigenetic inheritance as events in which information is transferred from parents to their offspring using various modifications of the chromatin structure. There can be changes in the structure of the DNA itself, but also of the proteins involved in the correct arrangement of the chromatin fiber. It is therefore not a classic heredity, which is based only on the sequence of the DNA itself inside the nucleus of the cell (Gallusci *et al*., 2023). Individual chromatin modifications that occur are collectively known as epigenetic markers and in certain cases can influence gene expression (Henderson and Jacobsen, 2007). Furthermore, they are irreplaceably involved in determining the function and cellular identity of the cell (Martin and Zhang, 2007). Unlike changes in the DNA sequence itself, i.e. mutations, all epigenetic changes are highly susceptible to the effects of the external environment and, if necessary, can be returned to their original state (Kakutani, 2002; Zhang *et al*., 2020). Thanks to this fact, we can study the influence of the external environment on gene expression over time, but epigenetics also contributes to a better understanding of how individual characteristics are passed from one generation to another (Henderson and Jacobsen, 2007).

Epigenetic modifications are mediated by many different mechanisms. Among the most studied are DNA methylation, histone modifications, and small non-coding RNAs (Henderson and Jacobsen, 2007; Gallusci *et al*., 2023). During the process of DNA methylation, a methyl group is added to a specific site, specifically a cytosine base. When this happens, for example, in the promoter part of a gene, the access of transcription factors and other proteins involved in gene transcription is prevented, and ultimately the gene itself is silenced. Histone modification is another well-known mechanism. Histones are protein structures around which the DNA molecule wraps and forms a so-called nucleosome (Klose and Bird, 2005). Several chemical reactions, such as acetylation, phosphorylation, or methylation can modify histones. These changes can affect the chromatin structure, which can result in changes in the expression of various genes (Zhang, 2003; Thiriet and Hayes, 2005; Martin and Zhang, 2005). The last of the mentioned are small non-coding RNAs. These molecules are capable of binding to mRNA and can cause their degradation or at least silencing, so the product in question will not be produced. It is therefore a mechanism that only works after the transcription of the gene (Henderson and Jacobsen, 2007). All the above-

mentioned mechanisms are involved in epigenetic inheritance and together form a very complex system.

2.2.2.1 Genomic imprinting

Genomic imprinting is the process by which genes are expressed differently based on whether they come from the maternal or paternal genome (Batista and Köhler, 2020). Unlike in insects, only individual genes are silenced in plants (Field *et al*., 2004; Wolff *et al*., 2011). In the case of plants, imprinting occurs only in flowering plants, specifically in the tissue of the endosperm and in a limited amount in the embryo itself (Wolff *et al*.,2011; Del Toro- De León *et al*., 2014). The whole process takes place thanks to epigenetic changes, so there are no changes to the DNA sequence. Specifically, methylation and demethylation of the maternal genome play the most important role here (Barlow, 1993; Barlow, 1994). When, even before fertilization, certain maternal genes are demethylated, they are subsequently expressed and the corresponding paternal genes are suppressed (Hsieh *et al*., 2011; Park *et al*., 2016). Tri-methylation of histone H3 at Lys27 has the opposite function, maternal genes marked in this way are subsequently suppressed and only paternal genes are expressed (Moreno-Romero, 2016; Hornslien *et al*., 2019).

2.2.3 Genome dominance

Genome dominance is another phenomenon in which the rules of Mendelian inheritance are violated. It is a case where one of the parental genomes exerts greater control over the phenotype compared to the other parental genome. It is particularly pronounced in allopolyploid organisms. This dominance can manifest in various aspects of the organism's phenotype, including growth characteristics, morphology, and physiological traits (Thomas *et al*., 2006; Glombik *et al*., 2020). Genome dominance in hybrid organisms, i.e. the differential contribution of parental genomes to the phenotype, is one of the key forces for adaptation to new environments, and diversification over evolutionary time. The study of this issue brings valuable information regarding the understanding of the evolutionary dynamics and adaptive potential of polyploid organisms (Schnable *et al*., 2011; Woodhouse *et al*., 2014; Glombik *et al*., 2021).

The process of genome dominance in hybrid organisms expresses itself at several levels, i.e. at the gene level, the chromosome level, and the genomic level (Glombik *et al*., 2020) The most common processes involved in the emergence of genome dominance at the genome level are changes in the composition and regulation of transposable elements (TEs) originating from different genomes (Freeling *et al*., 2012), large epigenetic changes across both genomes (Edger *et al*., 2017) and downsizing or amplification of some sections of genomes. (Glombik *et al*., 2020).

Following the changes at the genome level, significant changes also occur at the level of individual genes. Allopolyploids contain twice as many genes, so there is a conflict between the respective homoeologs. This conflict presents itself in the emergence of a number of genetic and epigenetic changes in genes. The accumulated changes are also called transcriptomic shock. As a result, several facts can occur. In the first case, one of the homoeologs will be completely silenced due to all the accumulated changes. The second possibility is that individual mutations cause a change in the function of one of the homoeologs. In the latter case, both homoeologs are disrupted and their resulting expression is comparable to the parental lines (Parisod *et al*., 2012; Yoo *et al*., 2013; Glombik *et al*., 2020). As a result, more dominant genome is the one in which the function of more genes has been preserved (Woodhouse *et al*., 2014). The issue of genome dominance at the chromosome level is discussed in more detail in the following section.

2.2.3.1 Non-mendelian chromosome segregation

Meiosis is a vital event in all living organisms. Chromosomes are segregated during meiosis. According to Mendel's laws, each allele, on a larger scale a chromosome, should have a 50% chance of being passed on to the next generation. However, there are exceptions in nature, when the given law is violated and the so-called non-Mendelian chromosome segregation occurs (Majka *et al*., 2023). This phenomenon is most often manifested in hybrid organisms when chromosomes are selected based on their parental origin (Henikoff *et al*., 2001) and, following this, their different behavior during meiosis (Majka *et al*., 2023). Several processes are likely involved, such as meiotic drive, variation in the proliferation of pollen tubes, germination, and fertility of pollen grains and seed yield (Glombik *et al*., 2020).

One of the hybrid organisms in which genome dominance at the chromosomal level has been studied is *Festulolium*. The *Lolium* genome predominates over the *Festuca* genome (Kopecký *et al*., 2006). The *Festuca* chromosomes are gradually replaced by those of *Lolium* in subsequent generations of the hybrid (Zwierzykowski *et al*., 2006; 2012). The stability of the genome composition differs in the hybrids depending on whether they are amphiploid or introgressed varieties. In the case of introgressed individuals, there is a complete elimination of the introgressed segments and a complete return to the original genotype (Kopecký *et al*., 2019). In amphiploid lines, most individuals are sterile, so it is difficult to assess the situation scientifically (Zwierzykowski *et al*., 2006).

2.2.3.1.1 Meiotic drive

The meiotic drive (Figure 1) occurs when a certain allele or other genetic elements can influence the process of meiosis in such a way that the transmission rate of the given allele to the next generation increases. These elements are called meiotic drivers. This usually occurs at the expense of alternative alleles (Lindholm *et al*., 2016; Clark and Akera, 2021; Majka *et al*., 2023). Changes in the transmission rate subsequently cause changes in the inheritance pattern i.e. specific alleles or entire segments of chromosomes are overrepresented in gametes (Lindholm *et al*., 2016). The meiotic drive is a result of various genetic mechanisms, including segregation distortion, in which alleles preferentially segregate into gametes during meiosis, or by interfering with the mechanisms of chromosome segregation during cell division (Lindholm *et al*., 2016; Kruger and Mueller, 2021; Majka *et al*., 2023).

The meiotic drive has so far been observed across various species from animals to fungi to plants (Buckler, 1999; Hurst and Werren, 2001; Courret *et al*., 2019). It can play a significant role in shaping genetic diversity, population dynamics, and evolutionary outcomes (Lindholm *et al*., 2016). It can serve as a driving force for non-Mendelian chromosome segregation.

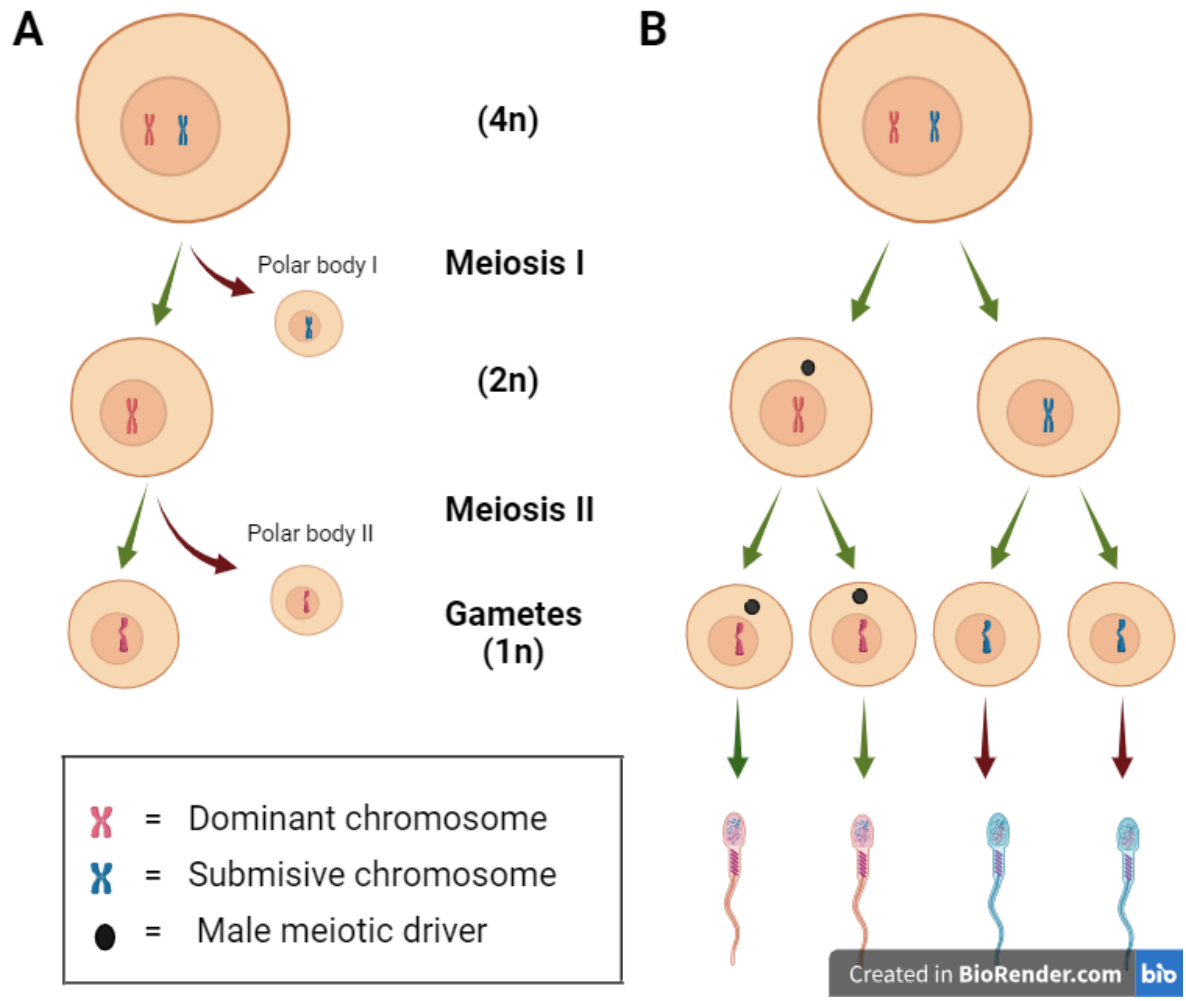

Figure 1: Schematic of meiotic drive. (A) Simplified model of female meiotic drive. The pink chromosome indicates the dominant chromosome, which is balanced and transferred to the egg cell thanks to the female meiotic drive. The blue chromosome represents the submissive chromosome, which is moved to polar bodies thanks to the female meiotic drive. (B) Simplified model of male meiotic drive. The black dot represents the male meiotic driver, which is located at favored (pink) chromosomes and can disrupt the development of gametes that carry the relevant competitive chromosome (blue). Red arrows induce decreasing in the fitness of sperm with the non-driving chromosome (blue). (Inspired by Kruger and Mueller, 2021), (Created with BioRender.com).

2.2.3.1.1.1 Female meiotic drive

During female meiosis, one oocyte undergoes two rounds of asymmetric cell division that give rise to one egg cell and 3 polar bodies, that are later degraded. The female meiotic drive can disrupt the segregation ratio of chromosomes and favor one genome over another to enter the egg cell leading to the dominance and submissiveness of individual parental genomes (Akera *et al*., 2017). The female meiotic drive has so far been studied in mouse hybrids

(Schuh and Ellenberg, 2008), monkeyflowers (Fishman and Kelly, 2015), *Allium cepa* and *Allium roylei* hybrids (Kopecký *et al*., 2022), and *Festulolium* (Majka *et al*., 2023).

2.2.3.1.1.2 Male meiotic drive

Several differences from female meiosis characterize male meiosis. The most striking is the fact that it is a symmetrical division, from which 4 gametes will emerge with an equal chance of fertilizing the egg. The mechanism of the male meiotic drive is therefore different from the female meiotic drive (Li and Dawe, 2009; Lukaszewski, 2010; Majka *et al*., 2023). Male meiotic drivers can act during or after meiosis. Drivers can disrupt the development of gametes that carry the relevant competitive allele. Through this mechanism, the total number of functional gametes is reduced and the driver thus gains a significant transmission advantage over competitive alleles (Srinivasa and Zanders, 2020; Courret *et al*., 2023).

The study by Majka *et al*. (2023) demonstrated that male meiosis contributes to non-mendelian inheritance in Festulolium plants. Large differences were noted in the binding of univalents originating from the *Festuca* and *Lolium* genomes to microtubules. Univalents of the dominant *Lolium* genome were correctly attached in 70.4%, while univalents of the submissive *Festuca* genome were correctly attached only in 19.3% and the respective *Festuca* univalents were more often eliminated. Together both female and male meiosis contribute to the emergence of genome dominance in *Festulolium* hybrids, but the mechanism is yet to be elucidated. The role of the meiotic drive in this process cannot be ruled out.

2.3 Kinetochores

The kinetochore is a large multiprotein complex found across all eukaryotic cells. It plays an irreplaceable role during meiotic and mitotic division when it serves as a connector between the chromosome at the centromere and the microtubules forming the dividing spindle. It aids in the correct attachment of the spindle, alignment of sister chromatids, and segregation itself. During the cell division, the correct attachment of the kinetochore to the microtubules is controlled by the spindle assembly checkpoint (SAC) which does not allow the division to proceed from metaphase to anaphase without the chromosomes being properly attached to the spindle (Cheeseman, 2014; Musacchio, 2015; Nagpal and Fukagawa, 2016).

2.3.1 Structure of kinetochores

The kinetochore complex (Figure 2) can be divided into two parts. Proteins and protein subcomplexes bound to or lying near the centromere are called the inner kinetochore and are constantly assembled throughout the cell cycle. The part that binds to microtubules is called the outer kinetochore and assembles only during mitotic or meiotic division. Even though the entire structure consists of a large number of proteins, it is a very dynamic system in which various changes and modifications occur all the time (Watts *et al*., 2018; Navarro and Cheeseman, 2021).

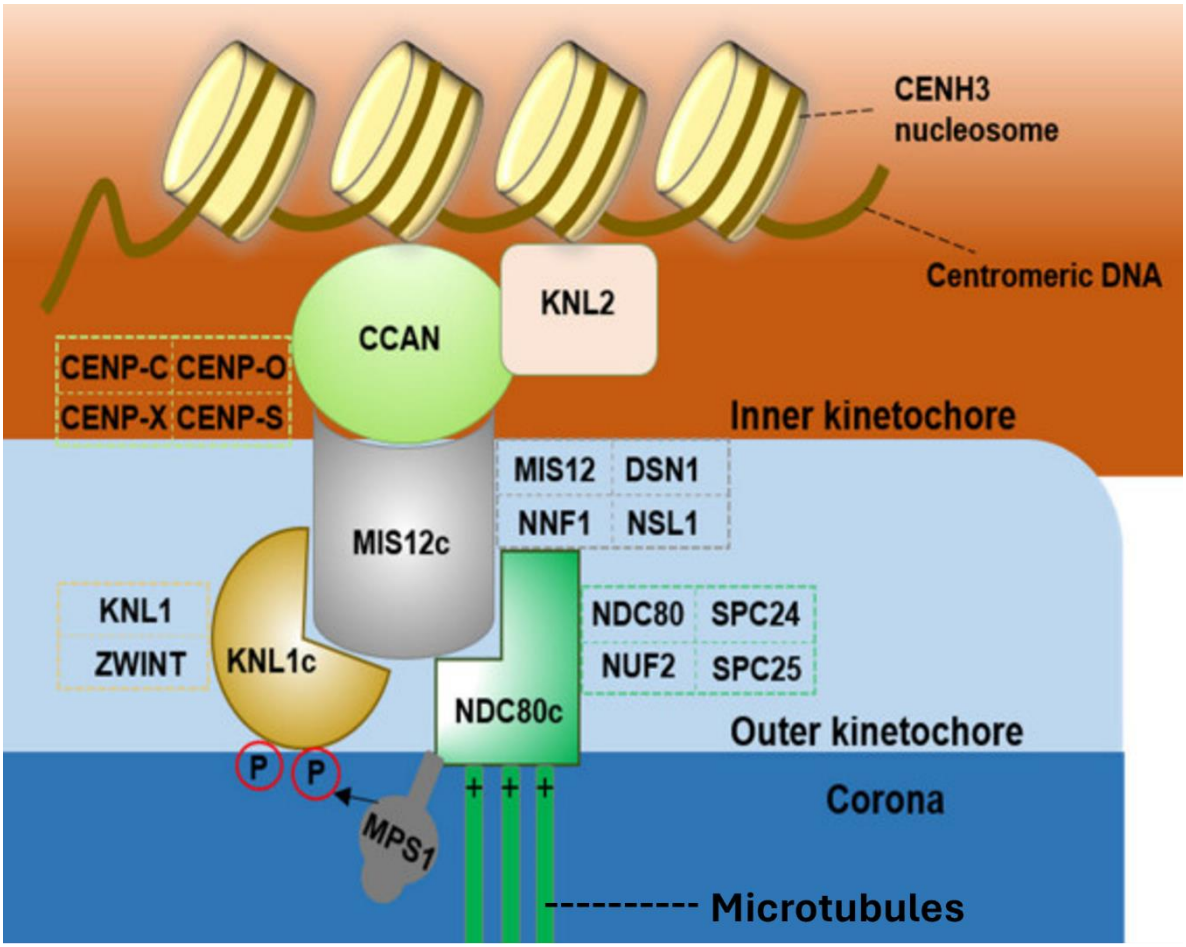

Figure 2: Model of plant kinetochore (Adopted from Zhou *et al.,* 2023).

2.3.1.1 Inner kinetochore

The inner kinetochore, generally abbreviated as CCAN (constitutive centromere associated network), consists of proteins and protein complexes that directly weigh into the centromere

or are located near it (Hori *et al*., 2008). The centromere region is a very specific place on the chromosome, which is specified by sequence-independent epigenetic markers, mainly by centromeric histone 3 (CENH3), (Zhou *et al*., 2023) in some organisms known as CENP-A (Régnier *et al*., 2005).

So far, 16 proteins that are part of CCAN have been discovered and described in vertebrates (Perpelescu and Fukagawa, 2011). However, only 4 of these 16 proteins were found in plants, CENP-C, CENP-O, CENP-S and CENP-X, but the last three were not localized on kinetochore (Kozgunova et al., 2019). Across all eukaryotic species, CENP-C is among the best-studied proteins. It was found that CENP-C is the only protein that directly interacts with CENH3 (Dawe *et al*., 1999) and at the same time with outer kinetochore protein complexes (Carroll *et al*., 2009; Kato et al., 2013). CENP-C is used to monitor the course of the cell cycle when the protein level remains the same almost throughout the cycle and increases when the cell enters mitosis. Similarly, the localization of CENP-C at the centromere changes during the cell cycle depending on the interaction partners (Fukagawa *et al*., 2001; Watanabe *et al*., 2019).

2.3.1.2 Outer kinetochore

The outer kinetochore can be divided into three sub-complexes: NDC80 complex, MIS12 complex, and KNL1 complex Together they are called the KMN network (Du and Dawe, 2007; Li and Dawe,2009; Su *et al*., 2021; Neumann *et al*., 2023). KMN serves as a bridge between microtubules and the centromere region of the chromosome with bound inner kinetochore proteins. In the plant kingdom, it has so far been confirmed that the KNL1 complex should co-localize with both the MIS12 and NDC80 complexes (Su *et al*., 2021). Furthermore, it has been shown in maize that during meiosis a connection between MIS12 and NDC80 complexes occurs (Li and Dawe, 2009).

2.3.1.2.1 MIS12 complex

So far, it has been shown that the MIS12 complex (Figure 3) of most eukaryotic organisms consists of 4 proteins MIS12, NNF1, DSN1, and NSL1. The DSN1 and NSL1 C-terminal domains are responsible for binding to the NDC80 complex (Petrovic *et al*., 2016, Dimitrova *et al*., 2016) and the NSL1 C-terminal domain itself also binds the KNL1 complex. Conversely, the N-terminal domains of MIS12 and NNF1 interact with inner kinetochore

proteins (Musacchio and Desai, 2017). In plants, only the MIS12 and NNF1 proteins have been further characterized (Li and Dawe, 2009; Allipra *et al*., 2022).

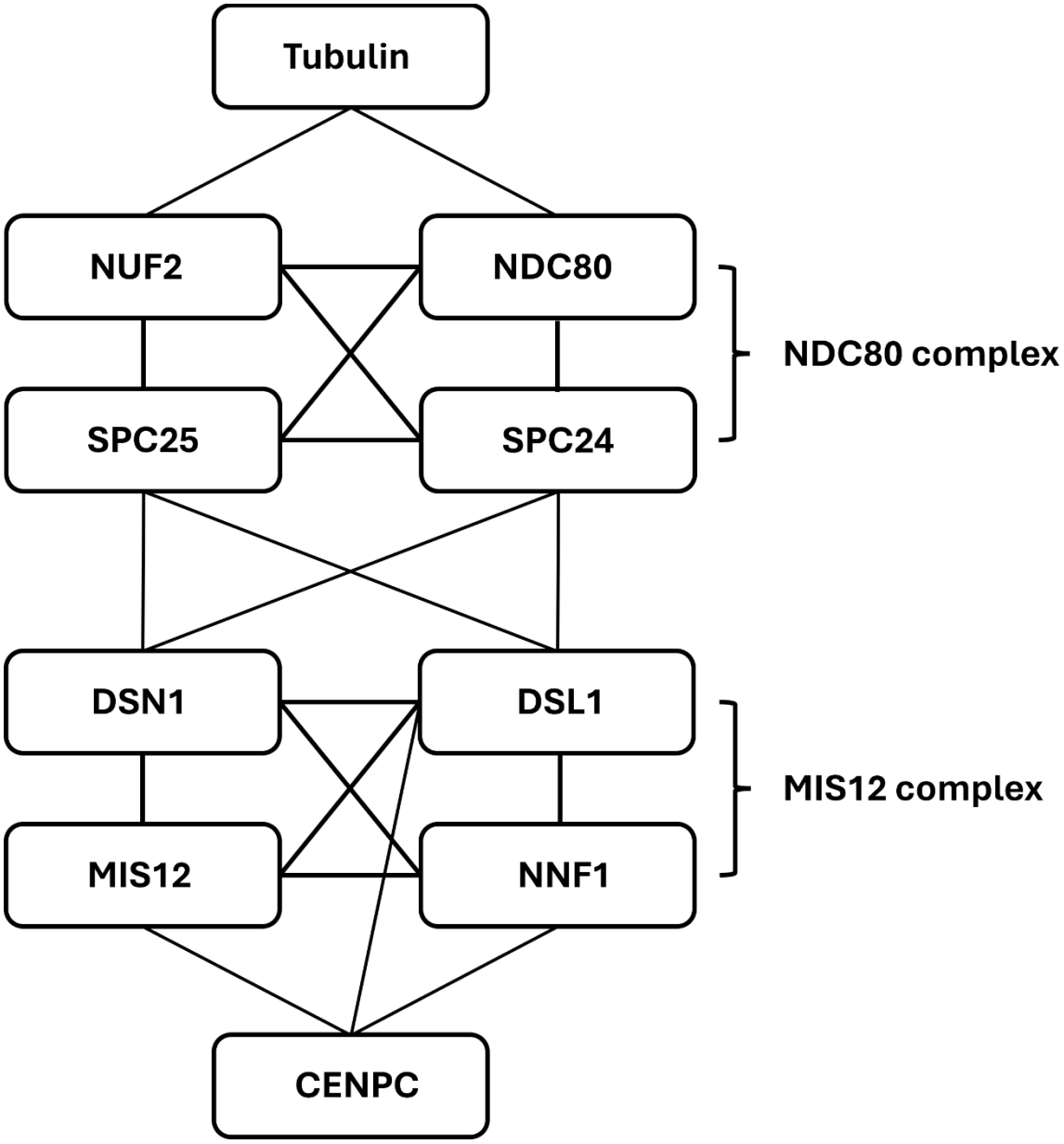

Figure 3: A putative model of the plant MIS12 and NDC80 complexes (Adopted from Neumann *et al.*, 2023).

2.3.1.2.1.1 NNF1

Necessary for nuclear function 1 (NNF1) is a kinetochore protein that is a part of the MIS12 complex of the outer kinetochore. NNF1 and its variants have so far only been well characterized in a small number of organisms, namely the yeast Saccharomyces cerevisiae,

the fruit fly Drosophila melanogaster, and humans. NNF1 ranks among the rapidly evolving kinetochore proteins when the percent amino acid substitution increases with the evolutionary distance (Blattner *et al*., 2017). In the plant kingdom, the protein has been best studied in the model plant *Arabidopsis thaliana* (AtNNF1). According to Allipra *et al*. (2022), in addition to the kinetochore function, AtNNF1 plays a role in polyamine and gibberellic acid metabolism and thus participates in plant growth and development.

2.3.1.2.2 NDC80 complex

The NDC80 complex (Figure 3) consists of a total of four kinetochore proteins NDC80, NUF2, SPC24 (MUN1), and SPC25 (Janke et al., 2001; Shin *et al*., 2018; Li *et al*., 2021). In the plant kingdom, the protein complex is best studied in the plant Arabidopsis thaliana (Shin *et al*., 2018; Li *et al*., 2021). It has been shown that in vertebrates, the NDC80 complex is formed by two heterodimers that are linked to each other. NDC80 and NUF2 together form the first heterodimer and are bound to microtubules by their N-terminal ends. The second SPC24-SPC25 heterodimer connects the NDC80 complex with the MIS12 complex with its C-terminal end (Ciferri *et al*., 2005; Wei *et al*., 2005).

2.3.1.2.2.1 NDC80

Like the entire NDC80 complex, the kinetochore protein itself, called Nuclear Division Cycle 80 (NDC80), has a highly conserved structure across all organisms (Wigge and Kilmartin 2001; Desai *et al*., 2003). Compared to NNF1, it is far more studied across species and is best characterized in yeast, humans, the frog *Xenopus laevis*, and others (Janke *et al*., 2001; Wigge and Kilmartin 2001; McCleland *et al*., 2003; Bharadwaj *et al*., 2004). Work by Du and Dawe (2007) showed that maize NDC80 is a constitutive kinetochore protein. It is found in high abundance in the G1 and S phases of the cell cycle and is associated with chromatin throughout. This association suggests an affinity of NDC80 for DNA itself or other DNA-associated proteins. Also, direct interaction between NDC80 and NUF2 proteins has been demonstrated in Arabidopsis thaliana (Li *et al*., 2021).

A previous work by Majka *et al*. (2023) outlined that the two kinetochore proteins NDC80 and NNF1 could play an important role in the process of genome dominance (see above) in *Festulolium* hybrid plants when some parts of the *Festuca*-derived genome are silenced including NDC80 and NNF1. Therefore these two proteins are expressed only from the

Lolium genome. This fact may result in an incomplete assembly of the kinetochore on Festuca chromosomes provided the structural differences between the homoeologues are large enough to influence their binding affinity. As a result, *Festuca* univalents do not attach to microtubules, are delayed during metaphase, form micronuclei, and are subsequently eliminated.

2.4 Protein-protein interactions

Proteins are classified as biopolymers; they are macromolecules consisting of proteinogenic amino acid chains of various lengths. They are one of the basic structural and functional units of living organisms and play an irreplaceable role during most processes inside cells, such as DNA replication, they form the "skeleton" of the cell itself, and they participate in the transport of various substances across the cell and between individual cells, they can serve as catalysts of various reactions and, last but not least, participate in signal transmission (Almeida, 2016)

All the above-mentioned processes, as well as the entire living organism, are, however, far more complex, and the entire process involves many individual proteins that interact with each other in various ways. The study of protein-protein interactions (PPIs), sometimes also referred to as interactomics, deals with this topic in more detail (Keskin *et al*., 2008). PPIs occur when at least two protein molecules physically join to form a protein complex. Hydrogen bonds, electrostatic forces, disulfide bonds, the hydrophobic effect, and other interactions hold together the entire complex (Tsai and Nussinov, 1997; Xu *et al*., 1997; Norel *et al*., 2001). As a rule, the connection occurs only at certain places of the protein molecule, the so-called domains, or binding domains of the protein (Thorn and Bogan, 2001).

2.4.1 Types of PPIs

There are several main criteria according to which we can divide PPIs or rather the resulting multiprotein complexes into the respective categories. The most basic way to distinguish protein complexes is by what subunits they are made of. If it is a group of identical subunits, we are talking about homo-oligomeric complexes. However, if there are several subunits of different kinds, we refer to them as hetero-oligomeric complexes (Goodsell and Olson, 2000).

The second criterion is the time for which the individual proteins in the complex remain connected. We are talking about transient and permanent complexes. Depending on the role of the complex inside the organism, the proteins can only be connected for a short time, while after fulfilling the necessary function, the complex disintegrates. For example, these are proteins involved in signal transmission or kinetochore proteins, and we are therefore talking about transient complexes. In the second case, proteins can interact with each other for a long time or even permanently, and therefore they are permanent complexes (Nooren and Thornton, 2003).

Another method of division is based on the covalency of the bonds connecting the proteins in the complex. Complexes connected mainly by disulfide bonds are referred to as covalent complexes, whereas if the connection is mediated by hydrogen bridges, Van der Waals forces and others, we are talking about non-covalent complexes (Westermarck *et al*., 2013). We can further divide protein complexes based on whether the complex is obligatory for the organism or not (Nooren and Thornton, 2003).

2.4.2 Functional domains

As already mentioned, the binding domains are responsible for the binding between the proteins of the complex. They are compact, spatially distinct unit. From an evolutionary point of view, domains can be perceived as parts of a certain protein sequence that are highly conserved across organisms (Pontig and Russell, 2002). Binding sites, due to their structure, serve as a recognition site for other proteins and subsequently for their mutual binding (Jones and Thornton, 1996). These sites are a maximum of 2000 Å2 (Chakrabarti and Janin, 2002). According to some studies, the binding between proteins does not involve the entire area, but only specific small parts of the sequence, so-called hotspots (Thorn and Bogan, 2001).

In some cases, a certain binding domain can recognize and bind only one type of protein, but this is not the case with a large group of domains, and the relevant site is recognized by several types of proteins that try to bind to the given site. The result of binding depends on various factors, such as the concentration of the given proteins in the environment, the conformation of molecules, environmental conditions (temperature, pH, ionic strength), post-translational modifications, allosteric regulation, and others (Keskin *et al*., 2008).

Following the information mentioned above, I would like to focus more on when certain amino acids change in the protein structure. This question is very complex, and it depends on the position where the amino acid is replaced and also which amino acid it is. If there is an exchange of similar amino acids, meaning amino acids with similar chemical and physical properties, the probability of changes in the protein structure is smaller (Dagan *et al*., 2002). Similarly, if the exchange of amino acids occurs outside the functional domains, the probability is also lower (Ung *et al*., 2006). The replacement can cause changes in the folding and stability of the protein (Lorch *et al*., 2000), change the function of the protein or its part (Tiede *et al*., 2006) and change the affinity to other proteins and other molecules (Ung *et al*., 2006) or there may be no changes at all.

2.5 Studied plants

2.5.1 Festuca pratensis

Festuca pratensis, commonly known as meadow fescue, is a cool-season perennial grass species that belongs to the *Poaceae* family. Native to Europe and Asia, it has become widely distributed across various continents due to its adaptability and use in agriculture. This grass is particularly well-suited for temperate climates and is often found in meadows, pastures, and along roadsides. It grows in bunches and the stalks reach a length of 40 to 120 cm, the root system is branched, but does not grow to a great depth. It is characterized by resistance to various abiotic stresses such as drought.

From an agricultural point of view, *Festuca* is among the most used fodder species. It is used to feed livestock, with the highest yield in the first three years. It is most often sown in combination with other species. In addition to agriculture, this grass is widely used in gardening and landscaping (Frame, 1991; Thomas *et al*., 2003; Straková *et al*., 2007).

2.5.2 Lolium multiflorum

Lolium multiflorum, also known as Italian ryegrass, is an annual or biennial plant from the *Poaceae* family. Like *Festuca*, it is a fodder plant grown mainly for cattle. It is characterized by the ability to produce many seeds and provides a high biomass yield. In addition to agriculture, it is also used in gardening and landscaping.

The plant originally comes from Europe, specifically from the countries of the temperate zone, but thanks to the above-mentioned properties, it has been spread all over the world. It prefers habitats with nutritious soil, it most often grows on borders and ruminants. As such, the plant reaches a size of 30 to 100 cm and grows in bunches. However, it is not as resistant as Festuca species, it is more demanding on nutrients, moisture, and temperature. Together with Festuca, they are widely used in grass breeding and serve as an important source of genetic material for interspecies hybridization (Yamada, 2005; Straková *et al*., 2007; Rognli *et al*., 2010).

2.5.3 Festulolium

Festulolium, a hybrid grass resulting from the crossbreeding of species from the *Festuca* and *Lolium* genera, combines the desirable traits of both parent plants. This artificial hybridization has led to the development of grasses that exhibit improved characteristics, such as increased yield, disease resistance, and adaptability to various environmental conditions. The crossing of these two species also occurs spontaneously in nature, but the resulting plants are sterile, so all available cultivars are artificially created. Like the *Festuca* and *Lolium* species, *Festulolium* is used in agriculture for sowing and processing as silage or hay, for livestock feed. It also contributes to the study and deeper understanding of plant biology, genetics, and molecular biology of grasses, as well as to the improvement and development of new techniques in the field of grass breeding and breeding in general (Humphreys and Harper, 2008; Kopecký *et al*., 2008; Kopecký *et al*., 2017; Kopecký *et al*., 2021).

3 Experimental part

3.1 Materials

3.1.1 Chemicals

Agarose (VWR), Agar powder (Himedia), Ambion nuclease-free water (Invitrogen), Complete supplement mixture (CSM), Drop-out : -His -Leu -Trp (Formedium), Complete supplement mixture (CSM), Drop-out : -Leu -Trp (Formedium), Cresol (Thermo Fisher Scientific), Dimethyl sulfoxide (Sigma Aldrich), Dreamtaq buffer 10x (Thermo Fisher Scientific), EDWARDS buffer, Ethanol 96%, Ethidium bromide (Sigma Aldrich), GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Fisher Scientific), Isopropanol, Lysogeny broth (LB), Lithium acetate (Thermo Fisher Scientific), PEG 3350 (Sigma Aldrich), Q5 High GC enhancer (New England Biolabs), Q5 reaction buffer (New Englang Biolabs), rCutSmart Buffer (New England Biolabs), Saccharose (Lach-ner), Salmon Sperm DNA solution (Invitrogen), SD Broth: 2% Glucose (Formedium), Silwet star (AgroBio Opava), Sodium hypochloride (Supelco), Substrate KTS 1 (Klasmann-Deilmann GmbH) TE buffer, TWEEN 20 (EMD Millipore corp.),YEB medium, YPD Broth (Formedium), 1x TBS buffer, 3,5-dimethoxy-4-hydroxyacetophenone (Sigma Aldrich), 3-amino-1,2,4-triazole (Sigma Aldrich), 5x T4 DNA Ligase Buffer (Thermo Fisher Scientific), 6x DNA Loading Dye (Thermo Fisher Scientific)

3.1.2 Chemical kits

BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), Gateway BP Clonase II Enzyme Mix (Invitrogen), Gateway LR Clonase II Enzyme Mix (Invitrogen), Gateway LR Clonase II Plus Enzyme Mix (Invitrogen), Phire Plant Direct PCR Kit (Thermo Fisher Scientific), QIAprep Spin Miniprep Kit (Qiagen), QIAquick PCR and Gel Cleanup Kit (Qiagen), RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), RNeasy Plant Mini Kit (Qiagen)

3.1.3 Antibiotics

Ampicillin (Fluka), Chloramphenicol (Serva), Gentamycin (Serva), HygromycinB (PAA), Kanamycin (Fluka), Rifampicin (Sigma), Spectinomycin (Sigma Aldrich), Timentin (Gold biotechnology Inc.), Zeocin (Invitrogen)

3.1.4 Enzymes

BP Clonase II enzyme, BsaI (New England Biolabs), DreamTag DNA polymerase (Thermo Fisher Scientific), Exonuclease I (Thermo Fisher Scientific), FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific), LR Clonase II enzyme, LR Clonase II Plus enzyme, Phire Hot Start II DNA polymerase (Thermo Fisher Scientific), Q5 High-Fidelity DNA Polymerase (New England Biolabs), RevertAid Reverse Transcriptase (Thermo Fisher Scientific), SacI (Fermentas), SphI-HF(New England Biolabs), T4 DNA ligase (Thermo Fisher Scientific)

3.1.5 Laboratory equipment

Automatic pipet E1-ClipTip (Thermo Scientific), Biological thermostat BT 120M (Labo MS spol. s.r.o.), Freezer box Jouan ULF 320 (MyBio), Fridge COG 2412 SA A (ARDO), Horizontal laminar flow box Biohazard (ThermoFisher Scientific), Ice maker GM 360 (NTF), Incubator Shaker Thermo Forma 420 (ThermoFisher Scientific), Laboratory scale AJ-820 CE (ViBRA), Macrovue Transilluminator LKB 2011 (Pharmacia LKB), Magnetic stirrer IKA RCT basic (IKA), Microcentrifuge MiniStar silverline (VWR), Phytotron chamber (Wiss Gallenkamp), Plant growth chambre (Percival), Printer UP-D897 (Sony), Qubid 3 fluorometer (Thermo Fisher), Refrigerated Centrifuge Multifuge X1R (Thermo Scientific), Refrigerated microcentrifuge Fresco 17 (Thermo Scientific), Semi-micro balances SBC 21 (SCALTEC), Single channel digital pipettes Nichipet EXII (Nichiryo), Spectrophotometer NanoDrop ND-1000 (Thermo Scientific), Thermocycler C100 Touch (Bio-Rad), Thermomixer C (Eppendorf), UV Transilluminator GVM30 GelVue (Syngene), Voltage source PowerPacTM (Bio-Rad), Vortex Reax control (Heidolph), Water bath SUB6 (Gran)

3.1.6 Software

BioRender, Geneious prime, Microsoft Excel, Microsoft Powerpoint

3.1.7 Plant material

Lolium multiflorum and *Festuca pratensis* plants grew for about 2 weeks in hydroponic conditions in a phytotron (16 hours, 20ºC day/8 hours, 16ºC night, humidity 60%). During growth, root tips were gradually cut for subsequent RNA isolation.

Arabidopsis thaliana ecotype Columbia 0 (Col-0) plants were grown in a phytotron (16 h, 19ºC day/ 8 h, 18ºC night, 60% humidity) until they were old enough for transformation (about 1,5 months) or their closed green flowers were removed for RNA isolation (roughly 4 weeks). The plants were grown in pots (size 6x6x7 cm) in soil optimal for plant growth (substrate KTS1).

3.1.8 Bacterial cultures

Escherichia coli strain TOP10, *Agrobacterium tumefaciens* strain GV3101.

3.1.9 Yeast cultures

Saccharomyces cerevisiae strain MaV203.

3.2 Methods

3.2.1 Isolation of RNA and generation of cDNA

RNA was isolated from root tips and leaves of *L. multiflorum* and *F. pratensis* and from flower buds of *A. thaliana*. The plant material was placed in 2 ml tubes together with glass beads and immediately frozen in liquid nitrogen. The tubes were placed in a homogenizer and the plant material was homogenized into a fine powder. The RNeasy Plant Mini Kit from Qiagen was used to isolate RNA from the homogenate and the procedure followed the manufacturer's instructions. The isolated RNA was then used for reverse transcription to generate cDNA. The RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific was used for this process. A cDNA concentration of 100 ng/µl was obtained, which was further diluted to a working concentration of 10 ng/µl and stored at -20ºC.

3.2.2 Primer design

Reverse and forward primers were designed for the *NDC80, SPC24, NUF2, NNF1, MIS12, CENPC, CENH3β* and *CENH3α* genes of *Festuca* and *Lolium* plants for their amplification

and later use in Yeast two hybrid method and for the *NNF1* gene of *A. thaliana* plants (Table 1) for their amplification and overexpression in *A. thaliana* plants. Whole genes were amplified, in the case of *MIS12* only functional domains were amplified and cDNA was used as a template. The resulting primers were between 18 and 26 base pairs in length. In the next step, attB overhangs were added to these primers so that they could be further ligated into entry vectors in the BP reaction of gateway cloning. All work was done in the computer program Geneious prime.

Table 1: List of used attB primers.

Name	**Sequence (5´ - 3´)**
At_NNF1 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGAAACCGGGTCATGAA
At_NNF1 R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTATATTGGAGGTAGCTTGTCTTTT
Fp_CENH3 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTCGCACGAAGCAC
Fp_CENH3 R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACCACCTTCGTCCCCCGAT
Fp_CENH3α F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTCGCACGAAGCACCCA
Fp_CENH3α R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACCACCTTGGCCCCCCGATA
Fp_CENPC F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCTCTGTCGACGCC
Fp_CENPC R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAATACTTTGCAACTTGAGCTAC
Fp_MIS12 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATACGAGAATGAGCAGGTGGGA
Fp_MIS12 R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTACCTGTACGTAGTTTGCCTTTCCAAG
Fp_NDC80 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTCAAATCTCCAAACCCCAACTC
Fp_NDC80 R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACTTCTTGTGTGAAGCCTCAGG
Fp_NNF1 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATTGCTGACCACCGATGGCGA
Fp_NNF1 R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCATTGTAGCTCCGACAGGCA
Fp_NUF2 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATTCGATGGCGTCCGGCTTC
Fp_NUF2 R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCATGTCCTTTTCCTAGCCCTTG
Fp_SPC24 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATGATGGCAGCACACGCTGGCA
Fp_SPC24 R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGTAGCAGGCCCTAAATCATTTTCC
Lm_CENH3 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTCGCACGAAGCAC
Lm_CENH3 R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACCACCTTCGTCCCCCGAT
Lm_CENH3α F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTCGCACGAAGCACCCA
Lm_CENH3α R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACCACCTTGGCCCCCCGATA
Lm_CENPC F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCTCTGTCGACGCC
Lm_CENPC R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAATACTTTGCAACGTGAGCTAC
Lm_MIS12 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGCAGCTCTTCATCAAC
Lm_MIS12 R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTACTCTCTTTGCAGTTCTTCAGATTCC
Lm_NDC80 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTCAAATCTCCAAACCCCAACTC

Lm_NDC80 R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACTTCTTGTGTGAAACCTCAGG
Lm_NNF1 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATAGCTGACCACCGATGGCGA
Lm_NNF1 R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCATTGTAGCTCCGACAGGCA
Lm_NUF2 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATTCGATGGCGTCCGGCTTC
Lm_NUF2 R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCATGTCCTTTTCCTAGCCCTTG
Lm_SPC24 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATGATGGCAGCACACGCTGGCA
Lm_SPC24 R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGTAGCAGGCCCTAAATCATTTTCC

3.2.3 Q5 polymerase chain reaction (PCR)

To amplify genes of interest, PCR with Q5 High-Fidelity DNA polymerase (New England BioLabs Inc.) was used. Annealing temperatures were optimized based on the company's website (https://tmcalculator.neb.com/#!/main) and they ranged from 72 to 63°C. The PCR reaction was carried out according to the manufacturer's instructions (Tables 2 and 3).

Table 2: Q5 PCR reaction mix.

Component	Volume (µl)	Final concentration
5x Q5 Reaction Buffer	5	1x
10 mM dNTPs	0,5	200 µM
10 µM Forward Primer	1,25	0,5 µM
10 µM Reverse Primer	1,25	0,5 µM
Template DNA (10 ng)	1	10 ng
Q5 High-Fidelity DNA Polymerase	0,25	0.02 U/µl
5X Q5 High GC Enhancer	5	1x
Nuclease-Free Water	To 25 µl	

Table 3: Q5 PCR cycling conditions.

Steps	Cycles	Temperatures	Time
Initial denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		72-63°C	30 seconds
Extension		72°C	30 seconds/kb
Final Extension	1	72°C	2 minutes

3.2.4 Gel electrophoresis

Q5 PCR products were visualized by gel electrophoresis. A 1.2% agarose gel with the addition of ethidium bromide (1 µl per 100 ml gel) in 1 x TBS buffer was used. 7 µl of 6x

Loading Dye was added to the 25 µl PCR reaction and the 25 µl was pipetted into the wells on the agarose gel. The GeneRuler 1 kb Plus DNA Ladder, which has a range from 75 to 20000 base pairs, was used as a marker. Electrophoresis was performed at 120 V for 60 minutes. After completion, the gel was visualized using the GeneSnap program.

3.2.5 Isolation of PCR product from agarose gel

Bands of the correct size were excised from the agarose gel under a UV lamp. The DNA was isolated using QIAquick PCR & Gel Cleanup Kit (Qiagen) and its concentration measured using NanoDrop One spectrophotometer.

3.2.6 Gateway cloning

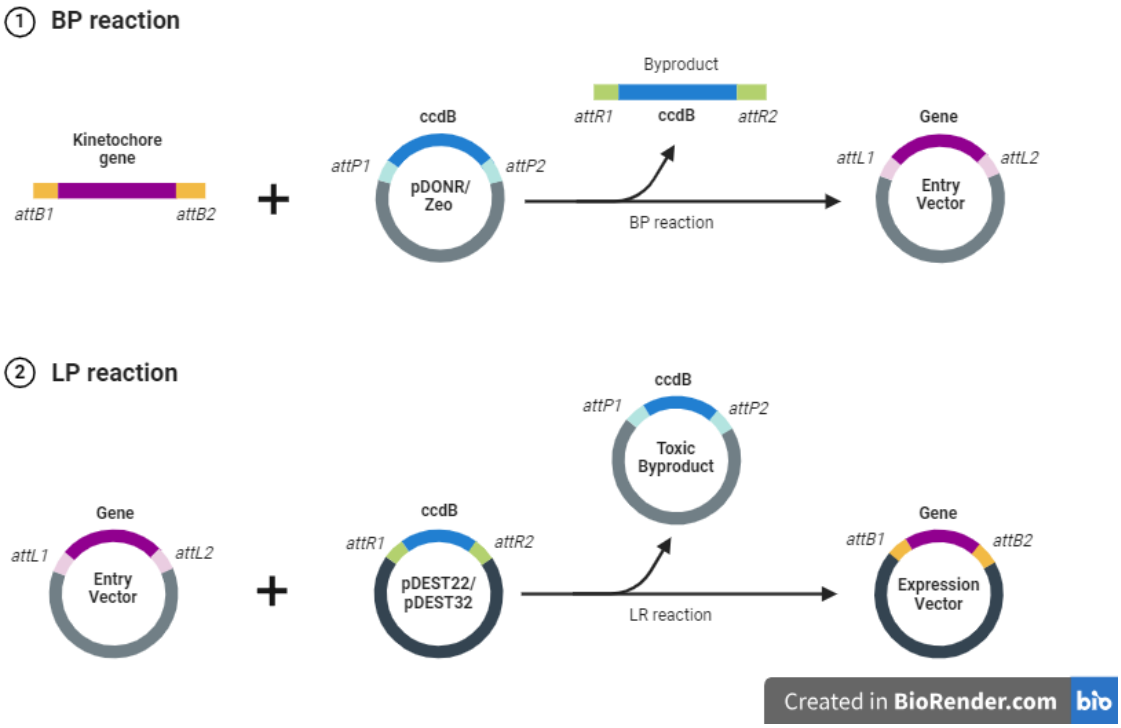

Figure 4: Workflow of Gateway cloning used to create expression clones for Y2H. (Created with BioRender.com)

3.2.6.1 BP reaction

PCR products with required attB overhangs were subsequently subjected to the BP reaction (Figure 4), the first phase of gateway cloning (Invitrogen), and introduced into the entry vector. In the case of the *Festuca* and *Lolium* genes, pDONR/Zeo (Appendix 1), carrying the

antibiotic zeocin resistance gene, was used as an entry vector. The pDONR207 (Appendix 2) vector with gentamicin resistance was used for *A. thaliana* genes. The entire BP reaction was carried out according to the manufacturer's instructions from Thermo Fisher Scientific.

3.2.6.2 TOP10 transformation

After the completion of the BP reaction, chemically competent bacteria *E. coli* (strain TOP10) was transformed with the entry clones. 3 µl of each construct was added to 50 ml of bacterial culture in a 2 ml tube and the whole mixture was allowed to stand on ice for 30 minutes. Subsequently, the bacteria were subjected to a temperature shock at 42ºC for 30 seconds and left on ice again for 2 minutes. 250 ml of LB medium was then added to the culture and the mixture was incubated on a shaker for 1 hour at 37°C and 225 rpm. After 1 hour, 100 µl of the bacterial culture was pipetted onto prepared Petri dishes with LB medium containing an appropriate antibiotic. The dishes were left in the incubator overnight at 37ºC.

3.2.6.3 Colony screen

Positive bacterial colonies were screened by PCR using the forward primer specific to the gene of interest and M13 reverse primer and DreamTag polymerase (Thermo Scientific™), (for conditions see Tables 4 and 5). The PCR products were visualized using gel electrophoresis as described above.

Table 4: DreamTag PCR reaction mix.

Component	Volume (µl)
10x DreamTaq buffer	2
10 mM dNTPs	0,4
10 µM Forward primer	1,25
10 µM Reverse primer	1,25
DreamTag polymerase	0,1
Cresol	4
Nuclease-Free Water	To 20 µl

Table 5: DreamTag PCR cycling conditions.

Steps	Cycles	Temperatures	Time
Initial denaturation	1	95ºC	3 minutes
Denaturation	30	95ºC	30 seconds
Annealing		55-65ºC	30 seconds
Extension		72ºC	1 min/kb
Final Extension	1	72ºC	10 minutes

3.2.6.4 Plasmid DNA isolation

Plasmid DNA was isolated from positive *E. coli* colonies using the QIAprep Spin Miniprep Kit (Qiagen). After isolation, the DNA concentration was measured on a NanoDrop spectrophotometer.

3.2.6.5 Sanger sequencing

Before proceeding to the next cloning step. Entry clones were checked by Sanger sequencing (Table 6). Each entry clone was sequenced using the forward primer specific to the gene of interest and M13 reverse primer. The amount of DNA added to the reaction was adjusted according to Table 7.

Table 6: Preparation of Sanger sequencing

Component	Volume (µl)
Buffer	1,5
BIG DYE	0,5
Primer (10µM)	1
DNA	2-3,5
Nuclease-free water	Add to 10 µl

Table 7: Amount of DNA for Sanger sequencing

Type of DNA	Amount of DNA (ng)
PCR product 200-1000 bp	5-20
PCR product 1000-2000 bp	10-40
PCR product over 2000 bp	40-100

3.2.6.6 LR reaction

Entry clones from the previous phase were subjected to the LR reaction (Figure 4) where the insert was transferred from the entry vector into the destination vector. In the case of *Festuca* and *Lolium* plants, genes were inserted into two vectors pDEST22 (Appendix 3) and pDEST32 (Appendix 4). The pDEST22 vector contains the gene for ampicillin resistance and carries the GAL4 DNA activation domain, whereas pDEST32 contains gentamicin resistance and the GAL4 DNA binding domain. Entry clones with *A. thaliana* genes were introduced into the multi-site gateway destination vector pH7m24GW-FAST (Appendix 5) together with pDONR P4P1r vector containing CaMV35S promoter (Appendix 6) available in the laboratory. The LR reaction was performed according to the manufacturer's instructions (Thermo Fisher Scientific). After completion of the LR phase, TOP10 transformation, colony screen of positive colonies, isolation of plasmid DNA, and Sanger sequencing were carried out as above.

3.2.6.6.1 Enzymatic digestion

When plasmids were re-isolated from already sequenced colonies, they were subjected to digestion with restriction enzymes to confirm their correctness. The appropriate mix (Table 8) was prepared, which was left for 1 hour at 37°C. Digestion was subsequently stopped by incubating the mixture at 65°C for 5 min. The cleavage result was visualized using gel electrophoresis (see subsection gel electrophoresis).

Table 8: Preparation of mixture for enzymatic digestion.

Component	Volume (µl)
Restriction enzyme	0,5
Buffer	3
Plasmid DNA	500 ng
Nuclease-free water	Add to 30 µl

3.2.7 CRISPR/Cas9

3.2.7.1 Oligo-sequence design

The most suitable guide sequences for Crispr-Cas9 (Figure 5) were selected using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR) and CHOPCHOP

(https://chopchop.cbu.uib.no/) for CENPC and NDC80 gene of *A. thaliana* plant (Table 9). A 5'-ATTG sequence was added to the designed forward primers at the beginning of the sequence, and a 5'-AAAC sequence was added to the reverse primer.

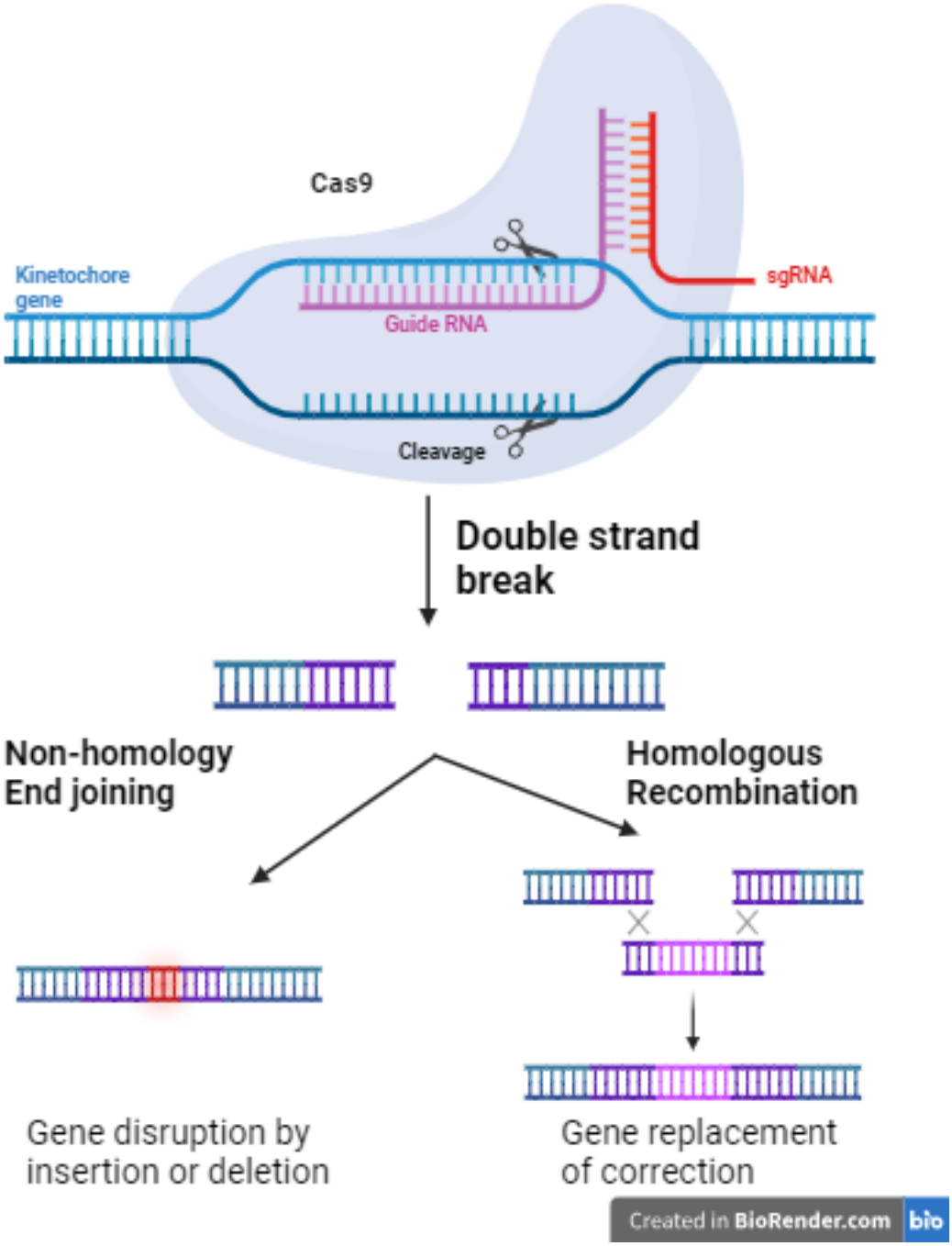

Figure 5: Schematic representation of mutation caused by CRISPR/Cas9 (Created in BioRender.com).

Table 9: List of used Crispr-Cas9 guide sequences.

Name	Sequence (5´ - 3´)
At_CENPC guide 1	CCGCTCGAATACTGGAGAGGTGA
At_CENPC guide 5	TTGAACATGCAGAGCACAAT
At_NDC80 guide 1	GGAGCTTCAGAACTTTAGCG
At_NDC80 guide 6	CCCTTGCGATTCCATCAAATGGG

3.2.7.2 Oligo-sequence annealing

A mixture for annealing was prepared (Table 10), which was subsequently incubated for 5 min at 95ºC and left for 20 min at room temperature to cool down.

Table 10: Mixture for oligo-sequence annealing

Component	Volume (µl)
Each oligo (100 µM)	1
Nuclease-free water	48

3.2.7.3 Vector digestion

The destination vector pAGM55273 (Appendix 7) was subjected to enzymatic digestion with BsaI restriction enzyme (Table 11). The resulting mixture was incubated for 1 hour at 37ºC. Subsequently, 1 µl of FastAP enzyme was added and the mixture was incubated for 10 min at 37ºC. The enzyme was inactivated at 75ºC for 5 min. The correct Digested vector was separated by gel electrophoresis. A band of the correct size was excised, the vector was isolated from the gel using the QIAquick PCR & Gel Cleanup Kit (Qiagen), and its concentration was measured using NanoDrop One spectrophotometer.

Table 11: Mixture for enzymatic digestion of destination vector.

Component	Volume (µl)
BsaI	0,5
Buffer	3
Vector DNA	500 ng
Nuclease-free water	Add to 30 µl

3.2.7.4 Ligation

A ligation mixture (Table 12) was prepared and incubated at room temperature for 1 hour. *E. coli* bacteria strain TOP 10 was transformed with the resulting construct and the remaining workflow is the same as mentioned above.

Table 12: Mixture for ligation.

Component	Volume (µl)
T4 ligase	1
5x ligase buffer	4
Digested vector DNA	3:1 pmol (vector : insert ration)
Insert DNA	
Nuclease-free water	Add to 20 µl

3.2.8 Transformation of *A. thumefaciens*

CRISPR/Cas9 and overexpressor clones were used to transform *A. tumefaciens* strain GV3101. 500 ng of plasmid DNA was added to 50 ml of chemically competent cells and left on ice for 15 min. Then the bacteria were snap frozen in liquid nitrogen and after thawing on ice, 1 ml of LB medium was added and the culture was incubated on a shaker at 28ºC, 225 rpm for 2 hours. After the incubation, 100 µl of the culture was pipetted onto prepared Petri dishes with YEB medium and the antibiotics rifampicin and kanamycin. The dishes were left in the incubator at 28ºC for 3 days.

3.2.9 *A. tumefaciens* mediated transformation using the floral dip method

A colony of *A. tumefaciens* carrying the desired construct was transferred to 5 ml of YEB medium with the antibiotics Rifampicin and Kanamycin and incubated overnight at 28°C and 225 rpm. The next day, 1 ml of the culture was transferred to 100 ml YEB medium with antibiotics. The culture was incubated overnight under the same conditions as above. On the third day, the entire volume of the grown culture was divided into two 50 ml flasks and centrifuged at 4500 rpm, 4ºC for 20 min. The pellet was resuspended in 5% sucrose solution and optical density (OD) at 600 nm was measured using a spectrophotometer. The desired OD value was 0,8. Silwet L-77 and a 100 mM solution of 4'-Hydroxy-3',5'-dimethoxyacetophenone (Acetosyringone) were added to the diluted culture (Table 13). Inflorescences of *A. thaliana* plants were immersed in the solution prepared above for 2-3

seconds. The plants were covered with a black bag and left in the phytotron. After 3 days, the bag was removed, and the plants continued to grow for 2 weeks in the phytotron until the seeds were harvested.

Table 13: Preparation of final solution for *Agrobacterium*-mediated transformation.

Component	Stock concentracion	Volume (µl)	Final concentracion	Final volume (ml)
Acetosyringon	100mM	400	200µM	200
Silwet L-77	100 %	100	0,05 %	

3.2.9.1 Sterilization of *A. thaliana* seeds

The harvested *A. thaliana* seeds were placed in a 50ml falcon with 75% ethanol and shaken for 5 minutes. The ethanol was poured off and 12.5% bleach solution with a drop of Tween 20 was added and the tube was again shaken for 20 minutes. Subsequently, the solution was poured off and the seeds were washed four times with sterile distilled water. After the last wash, the water was replaced with 0.5% agarose with the addition of the antibiotic kanamycin, and the seed in this solution was poured onto Petri dishes with ½ MS medium + 1% sucrose and Timentin and Kanamycin antibiotics.

3.2.9.2 Selection of transformed *A. thaliana* plants

The dishes with the sterile seed from the previous step were left in the fridge for 2 days and then were put in the phytotron (16 h, 19ºC day/ 8 h, 18ºC night, 60% humidity) for 10 days. Positive seedlings were subsequently selected (Positive seedlings had green cotyledons and negative seedlings had yellowing cotyledons.) from the dishes and transferred to pots (6x6x7 cm) with soil (Substrate KTS 1) and placed in the phytotron (16 h, 19ºC day/ 8 h, 18ºC night, 60% humidity).

3.2.9.3 DNA isolation using high throughput EDWARDS

3.2.9.3.1 Sampling

A small piece of the leaf (0.5-1.0 x 0.5 cm) was taken from each plant (about one-month-old) using scissors and tweezers and transferred to labeled 1.2 ml VWR Collection Microtubes containing small glass beads. The samples were immediately frozen in liquid nitrogen.

3.2.9.3.2 DNA extraction

Frozen samples were homogenized using a QIAGEN TissueLyser. The homogenization process took place for 1 min (frequency 30/s), then the sample was frozen again in liquid nitrogen and homogenized for another 1 min. 100 µl of EDWARDS buffer (Table 14) was added to the homogenate, vortexed for 15 s, and centrifuged at 2250 x g for 20 min at room temperature. 80 µl of supernatant was taken from each sample and transferred to a 96-well plate. Subsequently, 80 µl isopropanol was added, vortexed, and centrifuged (2250 x g, 10 min, room temperature). The supernatant was collected and 80 µL of 70% ethanol was added and centrifuged (2250 x g, 10 min, room temperature). Again, the supernatant was collected, and the excess ethanol was allowed to evaporate. In the final step, 50 µL of TE buffer was added and the samples were left overnight at 4°C to completely dissolve the pellet. Due to the lack of time, the procedure was carried out by the technicians.

Table 14: EDWARDS buffer (100 ml)

Component	Stock concentration (M)	Volume (ml)
Tris (pH 7.5 or 8)	1	20
NaCl	5	5
EDTA	0,5	5
SDS	10%	5
Water		65

3.2.9.4 Genotyping

DNA isolated by EDWARDS method was used for genotyping. In the case of *A. thaliana* knock-out lines, primers were designed (Table 15) to surround individual RNA guide sequences in the given gene. DNA fragments from individual plants were amplified and subjected to Sanger sequencing. The data were processed in the Geneious prime program.

3.2.9.5 PCR with casan primers

Plants of the T2 generation were screened for the presence of an active CRISPR/Cas9 cassette. DNA isolated from plants using the EDWARDS method was subjected to PCR (Table 16) with casan primers (Table 17). The empty vector pAGM55273 carrying the CRISPR/Cas9 cassette was used as a positive control.

Table 15: List of used guide primers.

Name	Sequence (5´ - 3´)
CENPC guide 1 F	ACTATTCTCTTGCAGGAAAAGC
CENPC guide 1 R	TCCAGGGGATGCATACTTTAT
CENPC guide 5 F	ATAGCATCCCGTATCAGCAA
CENPC guide 5 R	TCCGGAGCATTTTCTACTTGAA
NDC80 guide 1 F	ATGGAGAAAGTTGTGGAGGAGA
NDC80 guide 1 R	ATCTCTAGCAACCTCAGCATC
NDC80 guide 6 F	ATAACTTCCCGATCTCTATCCG
NDC80 guide 6 R	AATTGTGAGGCGTATTTGGAG

Table 16: PCR with casan primers cycling conditions.

Steps	Cycles	Temperatures	Time
Initial denaturation	1	95ºC	3 minutes
Denaturation	30	95ºC	30 seconds
Annealing		64,1ºC	30 seconds
Extension		72ºC	1 min/kb
Final Extension	1	72ºC	10 minutes

Table 17: List of sequences of casan primers

Name	Sequence (5´ - 3´)
Casan 2	CAGCTGCAGCTTCGCGTCCTC
Casan 3	GGCCGTTATCACCGATGAATACAAGG

3.2.10 Yeast two-hybrid (Y2H)

The method depends on the correct binding of the transcription factor to the upstream activation sequence and thus the production of the reporter gene will be triggered. In our work, we use the gene for the transcription factor Gal4, which is divided into two parts: DNA-binding domain (BD) and activation domain (AD). The destination vectors pDEST22 (referred to as the prey vector) and pDEST32 (referred to as the bait vector) each carry one domain. pDEST22 carries AD and pDEST32 carries BD. In our case, the *HIS3* gene is used as a reporter gene, which enables the yeast to survive on a selection medium without the amino acid Histidine. 3-amino-1,2,4-triazole (3AT) is added to the selection medium, which serves as a competitive inhibitor of the *HIS3* gene. Therefore, only cells with sufficient expression of the *HIS3* gene will survive. Therefore, the presence of 3AT will select clones

with high levels of *HIS3* which depends on the strength of the interaction between bait and prey.

3.2.10.1 Yeast transformation

Yeast strain Mav203 was co-transformed with two expression clones (Table 18), pDEST32 bait vector containing the gene of interest GAL4 binding domain and pDEST22 prey vector containing GAL4 activation domain. 100 ml of YPD medium was first inoculated with a fresh yeast colony and incubated at 30°C and 160 rpm overnight to the desired OD600 between 1 and 1.5. The culture was divided into two 50 ml flasks and centrifuged at 4000 rpm, for 5 min. The supernatant was discarded, and the pellet was washed twice with 20 mL of 0.1 M lithium acetate (LiAc) solution. After washing, the pellet was resuspended in 2 ml of LiAc and incubated for 1 hour at 160 rpm and 30°C. A transformation mix (Table 19) was prepared, which was left at 30ºC for 30 min in a water bath and the tube was mixed by inversion every 10 min. Subsequently, the cells were heat shocked for 5 minutes at 42ºC, followed by 3 min on ice. The solution was centrifuged for 1 min at 10000 rpm, the supernatant was discarded, and the pelt was resuspended in 110 µl of sterile water. 100 µl was pipetted onto Petri dishes with Synthetic Defined lacking -Leucine/-Tryptophan medium and allowed to incubate for 3 days at 28ºC.

3.2.10.1.1 Y2H

Individual yeast colonies co-transformed in the previous step were resuspended in 500 µl of sterile water and pipetted in three replicates onto Synthetic Defined -Leucine/-Tryptophan/-Histidine selection medium with varying concentrations of 3 amino-1,2,4-triazole (3-AT), (Table 20) and incubated for 3-4 days at 28ºC. After incubation, the image material of individual samples was taken. All proteins were cloned and tested as both bait and prey. Proteins of the same parental origin were used as positive controls and autoactivation between the empty vector and the vector carrying the construct was also investigated. Interactions were investigated between MIS12 and NNF1 and NDC80 and NUF2 proteins.

Table 18: Table of combinations of constructs for Y2H.

Activation Domain (AD) (Constructs with pDEST22)	DNA-binding Domain (BD) (Constructs with pDEST32)
pDEST22	pDEST32
LmMIS12	LmNNF1
LmMIS12	pDEST32
pDEST22	LmNNF1
LmNNF1	LmMIS12
pDEST22	LmMIS12
LmNNF1	pDEST32
FpMIS12	FpNNF1
FpMIS12	pDEST32
pDEST22	FpNNF1
FpNNF1	FpMIS12
pDEST22	FpMIS12
FpNNF1	pDEST32
LmMIS12	FpNNF1
FpNNF1	LmMIS12
FpMIS12	LmNNF1
LmNNF1	FpMIS12
LmNUF2	LmNDC80
LmNUF2	pDEST32
pDEST22	LmNDC80
LmNDC80	LmNUF2
pDEST22	LmNUF2
LmNDC80	pDEST32
FpNUF2	FpNDC80
FpNUF2	pDEST32
pDEST22	FpNDC80
FpNDC80	FpNUF2
pDEST22	FpNUF2
FpNDC80	pDEST32
LmNUF2	FpNDC80
FpNDC80	LmNUF2
FpNUF2	LmNDC80
LmNDC80	FpNUF2

Table 19: Mix for the transformation of yeast

Component	Volume
Plasmid (with pDEST22)	1 µg
Plasmid (with pDEST32)	1 µg
Cell culture	150 µl
50% PEG 3350	350 µl
Salmon sperm DNA	2 µl

Table 20: Preparation of media with different concentration of 3-AT

Stock concentration of 3-AT	Volume of 3-AT	Final concentration of 3-AT	Final Volume
1 M	0 µl	0 mM	25 ml
	250 µl	10 mM	
	625 µl	25 mM	
	1250 µl	50 mM	
	1875 µl	75 mM	
	2500 µl	100 mM	

4 Results

4.1 Knock-out lines of *A. thaliana*

In the second part of the thesis, the goal was to generate two konck-out lines of *A. thaliana* for the kinetochore gene *CENPC*, and *NDC80* respectively. The lines were created using the CRISPR/Cas9 method. The transgenic lines were genotyped by Sanger sequencing.

4.1.1 *A. thaliana CENPC* knock-out lines

Two guide RNAs (guide1 and 5) were designed for the *CENPC* gene and cloned into the pAGM55273 binary vector containing the multi-intron Cas9 gene (Grützner *et al*., 2020). Using Agrobacterium-mediated transformation, two *A. thaliana* plants were transformed for each construct. The seed was subjected to selection on kanamycin-containing selection media. After selection, I received 2 plants for guide 1 and 5 plants for guide 5. However, the results of Sanger sequencing, showed that none of the plants carried any modification in the CENPC gene at the respective location of the guide sequence.

4.1.2 *A. thaliana NDC80* knock-out lines

To knock-out *NDC80* gene, 2 guide RNAs (1 and 6) were used. As previously, two *A. thaliana* plants were transformed for each construct. After selection, I received 20 plants for guide 1 and 20 plants for guide 6. Sanger sequencing results indicated a change within the guide 1 sequence in 9 plants (Figure 6A; Appendix 7) out of 20. In most lines, a certain amount of nucleotides was deleted in the guide sequence (lines 2, 4, 7, 10 and 20). In line 15 and 11, on the contrary, one nucleotide was inserted. The majority of lines further displayed nucleotide substitutions (lines 9, 10, 12, 15 and 20). All of these changes affected the amino acid sequence (Figure 6B; Appendix 7) but, in the majority of cases, did not disturb the reading frame (lines 2, 4, 7, 9, 10 and 20). Lines 11, 12 and 15 showed a reading frame shift and had a premature stop codon introduced.

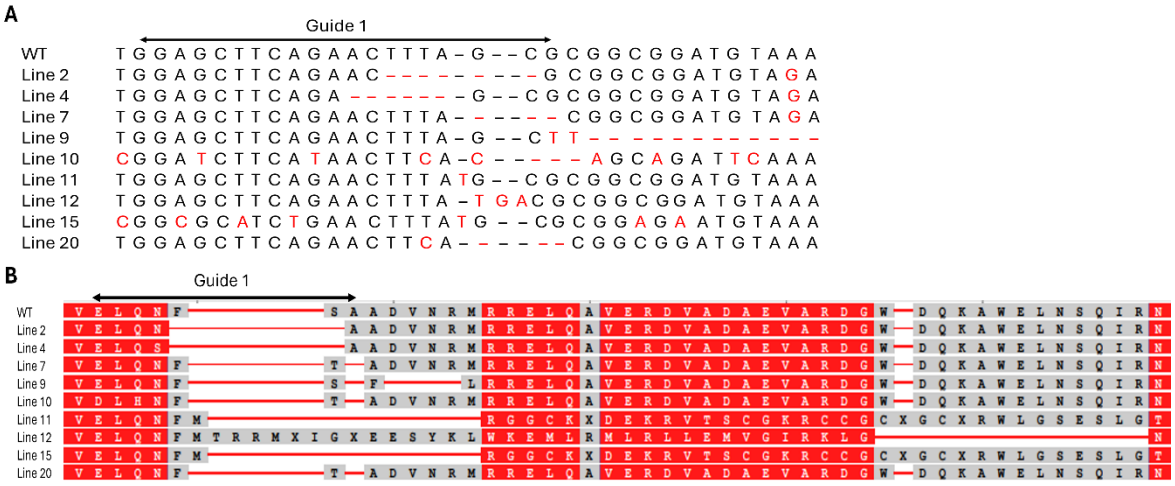

Figure 6: Comparison of the wild-type sequence and the sequence of plant lines with a change in the Guide 1 sequence. (A) Sanger sequencing results in positive lines of the T1 generation with a change in the Guide 1 sequence. (B) Effect of changes in nucleotide sequence on amino acid sequence in positive lines of the T1 generation. X = stop codon.

In the case of guide 6 lines, the sequence change was confirmed in 8 plants (Figure 7A; Appendix 8) out of 20. Compared to guide 1 lines, in most cases there were only point mutations of individual nucleotides. Only in line 3 was a large insertion of 39 nucleotides to the guide sequence. This led to the induction of 13 new amino acids into the reading frame, which was not changed in any way except for the aforementioned addition. In lines 1, 6, 8 and 9, one or two amino acids were substituted but these changes did not change the reading frame of the protein. However, changes in the nucleotide sequence were significantly manifested in lines 2, 11 and 20, where in all cases the reading frame was completely different from the wild-type and several premature stop codons were introduced in the amino acid sequence (Figure 7B; Appendix 8).

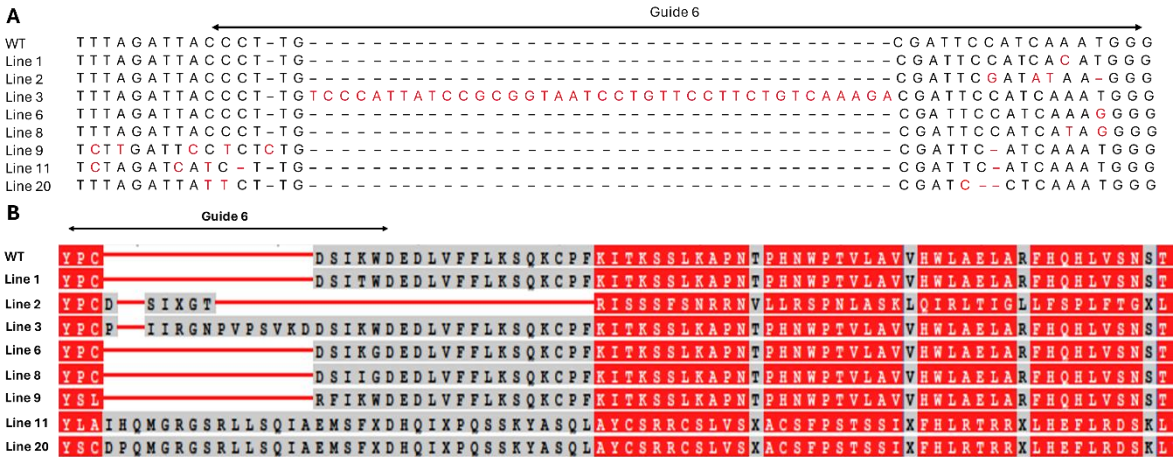

Figure 7: Comparison of the wild-type sequence with the sequence of plant lines with a change in the Guide 6 sequence. (A) Sanger sequencing results in positive lines of the T1 generation with a change in the Guide 1 sequence. (B) Effect of changes in nucleotide sequence on amino acid sequence in positive lines of the T1 generation. X = stop codon.

4.1.2.1 Selection of plants in second generation

Seeds were obtained from all plants of the T1 generation that showed a change in the guide sequences. The most promising lines from the T1 generation were selected for further work, namely lines 9 and 11 with a change in guide 1 sequence and lines 2, 3, and 11 with changes in guide 6. A total of 40 plants were grown for each line, which were subsequently tested by Sanger sequencing, to find out whether they inherited the appropriate modifications in the guide sequence. At the same time, all plants were screened using PCR with Cas9-specific primers to find out whether the CRISPR/Cas9 cassette was still active in the plants or not. In T2 generation plants, the cassette should be inactive to prevent further modifications to the genome.

For the plant to be selected for further experiments, it had to meet several parameters. The first was that it had to carry the appropriate modification in the guide sequence. The second parameter was the fact that the plant had to carry the respective modification in a heterozygous state, i.e. that it carried one wild-type allele of the *NDC80* gene and the other modified one, because, according to our hypothesis, homozygotes carrying two modified alleles should not be able to survive. The last parameter was that the respective plant must not carry an active cassette for CRISPR/Cas9.

Based on the selected parameters, we managed to obtain only two positive plants in the T2 generation. These were plants 11 and 12, which had respective changes in the guide 1 sequence and were derived from line 11 of T1 (Figure 8A; Appendix 9). Based on the Sanger sequencing results, both plants were heterozygous for the mutation. PCR results with casan primers confirmed that the plants do not contain an active CRISPR/Cas9 cassette (Figure 8B). In the case of the other examined lines, we did not receive unequivocally positive plants.

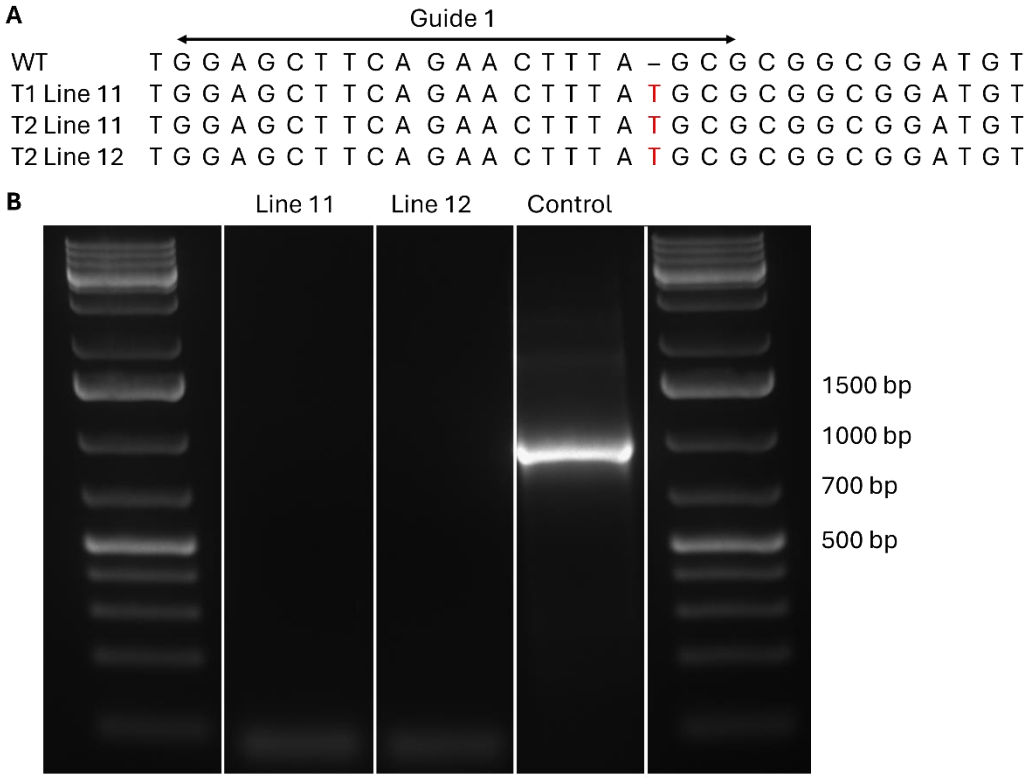

Figure 8: Results of screening of positive lines of the T2 generation (A) Comparison of the nucleotide sequence of the wild-type plant, maternal line 11 from the T1 generation guide 1 and two lines from the T2 generation (lines 11 and 12). (B) Results of the PCR with casan primer. The first and last column is The GeneRuler 1 kb Plus DNA Ladder; 2nd column = T2 line 11; 3rd column = T2 line 12; 4th column = positive control (empty vector pAGM55273).

4.2 Interactions of kinetochore proteins

It is a generally accepted and proven fact that across eukaryotic organisms interactions between the kinetochore proteins MIS12 and NNF1 and NDC80 and NUF2 occur during kinetochore assembly during cell division (Shin *et al*., 2018; Li *et al*., 2021; Allipra *et al*., 2022). Our first aim was to show whether the respective pairs of kinetochore proteins derived

from *F. pratensis* and *L. multiflorum* would interact with each other in Y2H assay and whether the strength of the interaction differs between protein variants derived from a single species and across species.

Using the gateway cloning method, the constructs carrying the kinetochore genes were inserted into two vectors, pDEST22 (prey vector) and 32 (bait vector), used in subsequent Y2H. Yeast was grown on selection media (SD medium without the addition of Histidine, Leucine and Tryptophan) with the addition of different concentrations of 3-AT. 3-AT serves as an inhibitor of the HIS3 gene, which should only be carried by transformed yeast colonies, and is used for more advanced selection of clones with strong interactions (Durfee *et al*., 1993). The constructs were tested in all combinations and the strength of autoactivation was checked for each construct. The term autoactivation means that the respective protein can trigger the activation of the HIS3 gene without having to interact with a second binding protein. For correct results, the auto-activation level should be very low to none.

4.2.1 Interactions of MIS12 and NNF1

First, interactions between proteins originating from the same organism were investigated. Yeast cells co-transformed with empty vectors (bait+prey) were used as a negative control. Two positive controls were also used. The first was auxin-responsive transcription factor (ARF) interacting with gibberellic acid insensitive transcription factor (GAI; Hu *et al.,* 2018; 2022) and the second was GAI interacting with Arabidopsis response regulator transcription factor (ARR; Rosa *et al.,* 2015). In *Lolium* (Figure 9; Appendix 10) interactions, there was a strong autoactivation of the LmMIS12 protein in both variants (bait and prey), on the contrary, the LmNNF1 protein showed very weak autoactivation as prey and no autoactivation as bait. The interaction between LmNNF1 prey and LmMIS12 bait was very strong, and in the opposite combination, the strength of the interaction was moderate but in neither case the interaction was not stronger than the autoactivation. Therefore, these results are inconclusive.

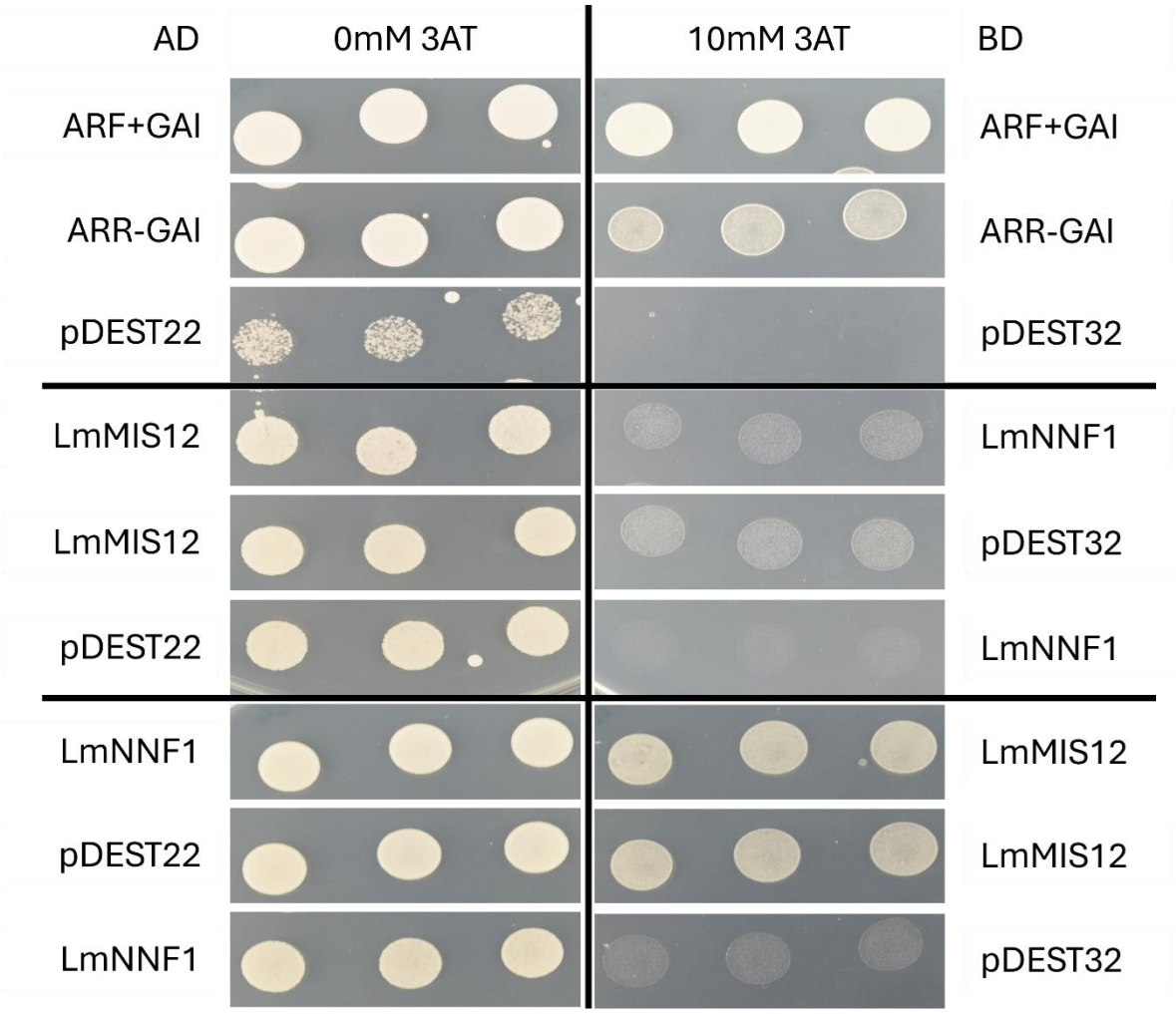

Figure 9: Y2H assay of interactions of *L. multiflorum* proteins LmMIS12 and LmNNF1 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).

Festuca proteins (Figure 10; Appendix 11) showed a high level of autoactivation of FpNNF1 in both combinations. For the second protein FpMIS12, autoactivation was very weak in both combinations. However, the resulting interactions of these two proteins together turned out to be very weak, especially compared to those of *Lolium*.

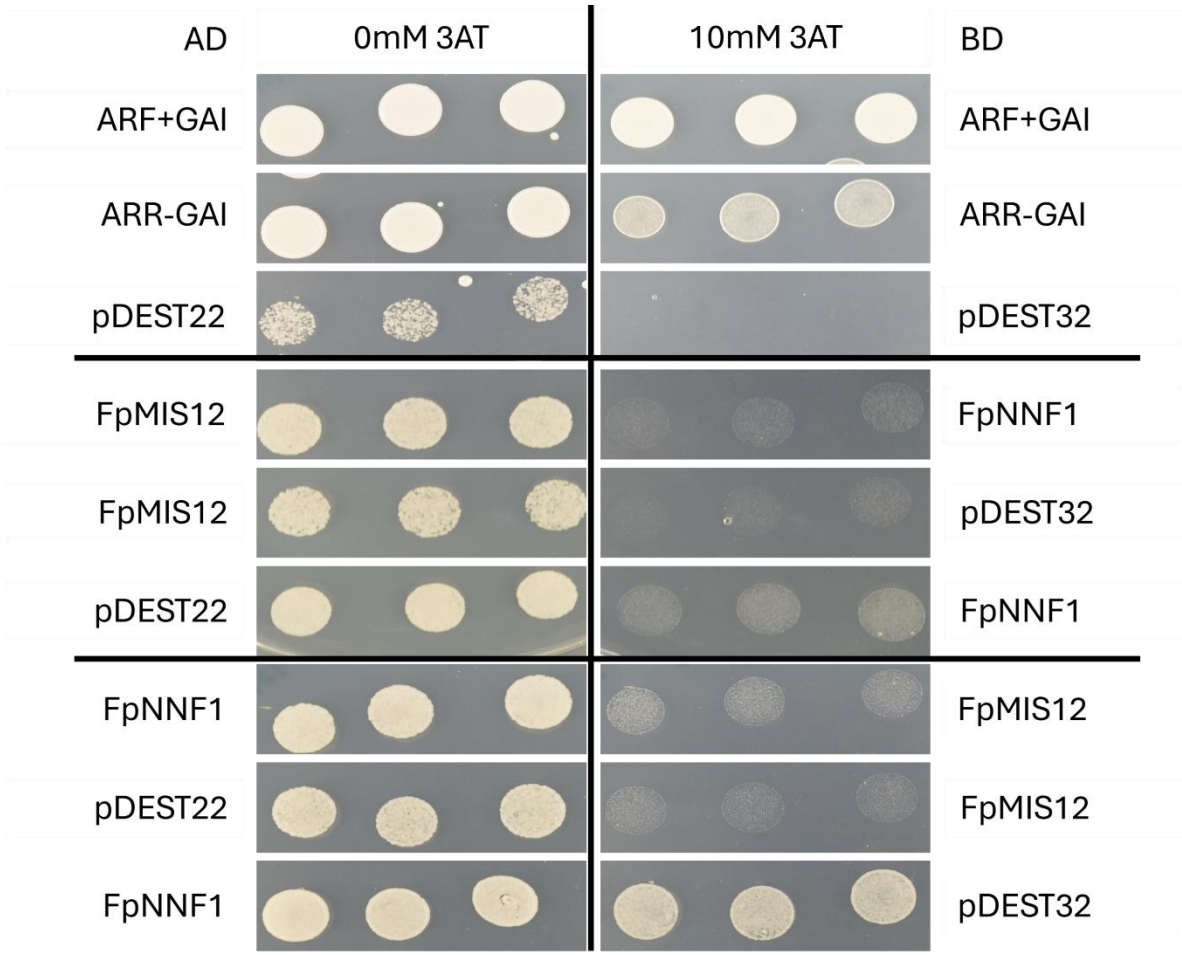

Figure 10: Y2H assay of interactions of *F. pratensis* proteins FpMIS12 and FpNNF1 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).

In the next step, differences in the strength of interactions between homoeologous proteins from Festuca and Lolium were investigated (Figure 11, Appendix 12). The weakest interaction showed FpMIS12 and LmNNF1 in both combinations. The strength of the interaction was also very weak for LmMIS12 as prey and FpNNF1 as bait. As mentioned, the interaction between the pair of Festuca proteins was also low. On the other hand, the pair of Lolium proteins showed the strongest interactions. A strong interaction was also shown by FpNNF1 as prey and LmMIS12 as bait. Taking into account the outcome of autoactivations, the results should be inconclusive.

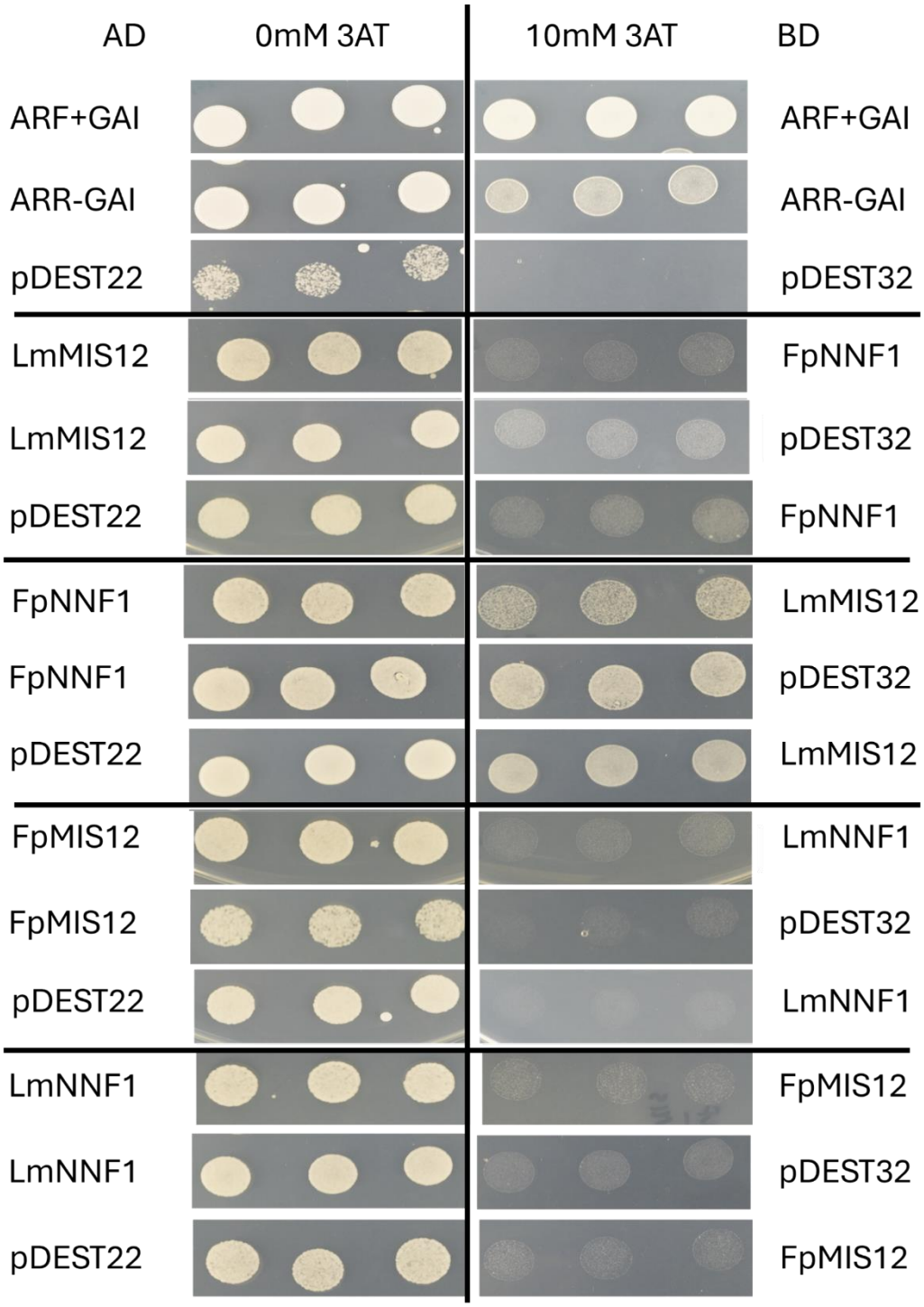

Figure 11: Y2H assay of interactions of homoeologs of MIS12 and NNF1 of *L. multiflorum and F. pratensis*. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).

4.2.2 Interactions of NDC80 and NUF2

Again, mutual interactions of proteins originating from the same species were first investigated, and whether autoactivation occurs in individual constructs. The same controls as above were used. In the case of Lolium proteins LmNDC80 and LmNUF2 (Figure 12; Appendix 13), high autoactivation was noted when they were used as bait. In the opposite cases, autoactivation did not occur in either of them. The strength of interaction between LmNDC80 and LmNUF2 was practically identical in both combinations but was equal to autoactivation.

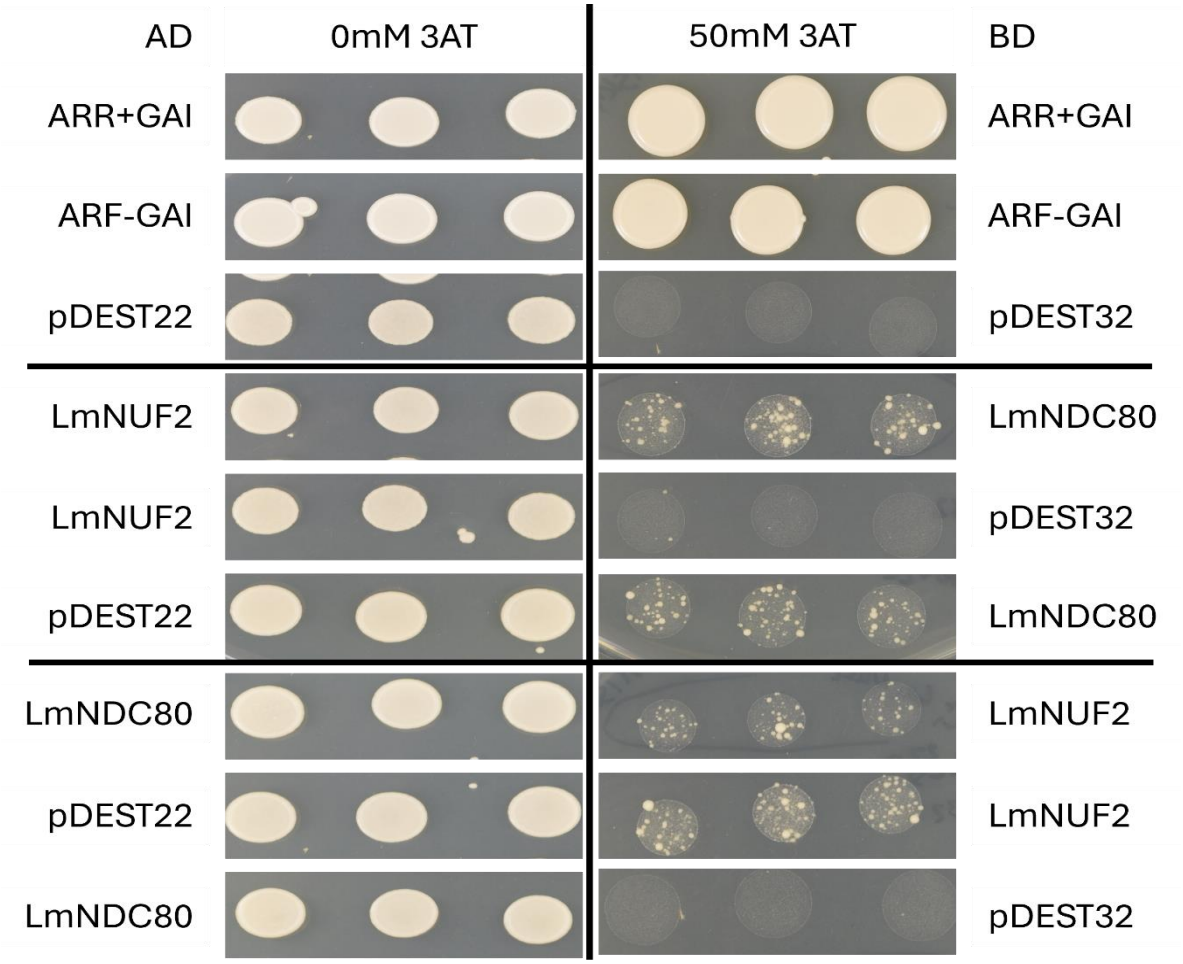

Figure 12: Y2H assay of interactions of *L. multiflorum* proteins LmNDC80 and LmNUF2 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).

As before, *Festuca* proteins FpNDC80 and FpNUF2 (Figure 13; Appendix 14) showed increased autoactivation when the respective constructs were present as bait. The highest autoactivation was generated by FpNDC80 as bait. As a prey, there was no autoactivation of the constructs. FpNUF2 as prey and FpNDC80 as bait interacted most strongly. In the opposite combination, the interactions were moderately strong. However, the results are greatly influenced by high autoactivation.

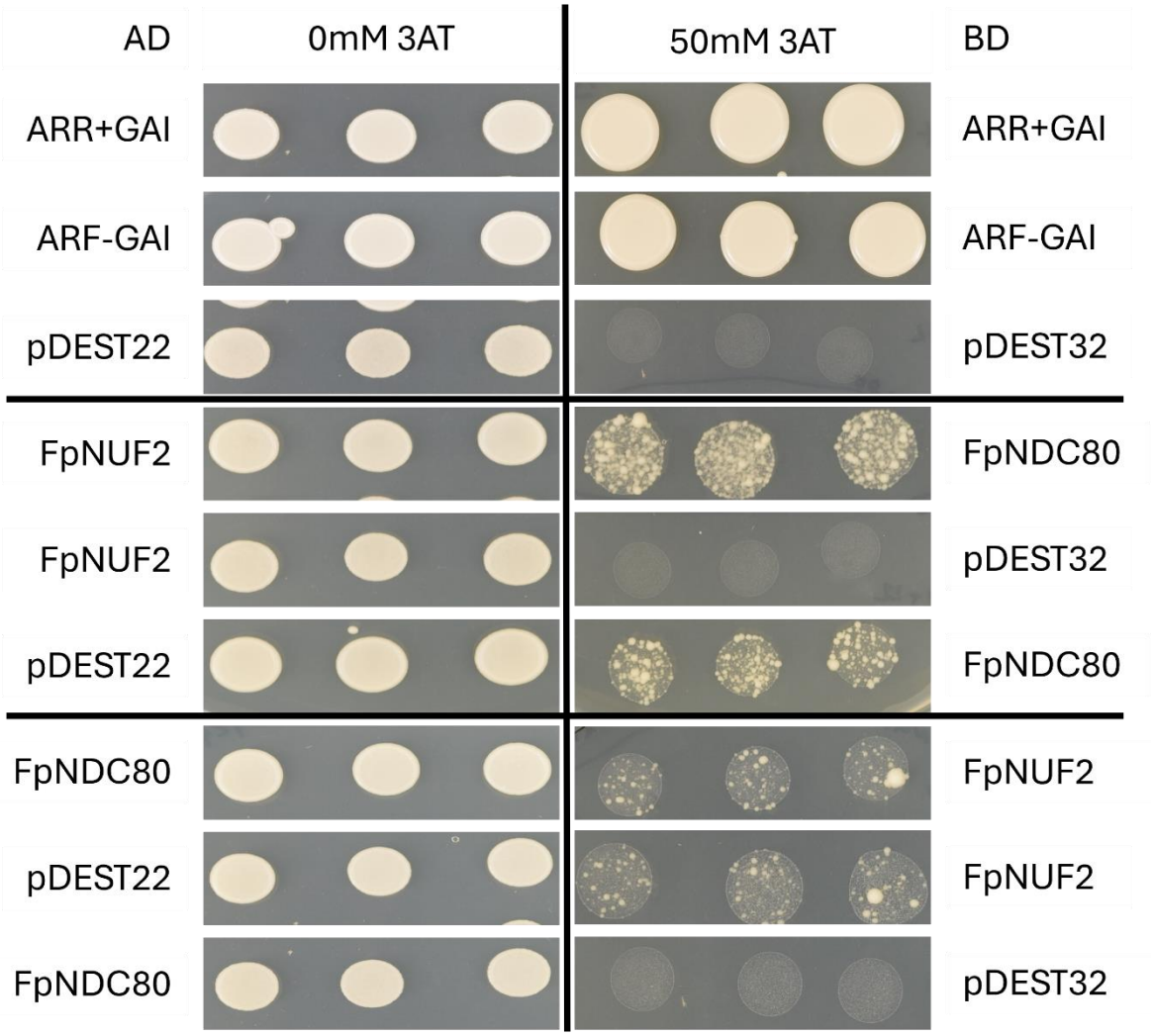

Figure 13: Y2H assay of interactions of F. pratensis proteins FpNDC80 and FpNUF2 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).

Subsequently, we checked whether we could find any differences in single species and interspecies interactions of individual proteins (Figure 14, Appendix 15). But given that all resulting interactions matched the strength of the respective auto activations. Thus, we cannot derive any provable conclusions from the data obtained.

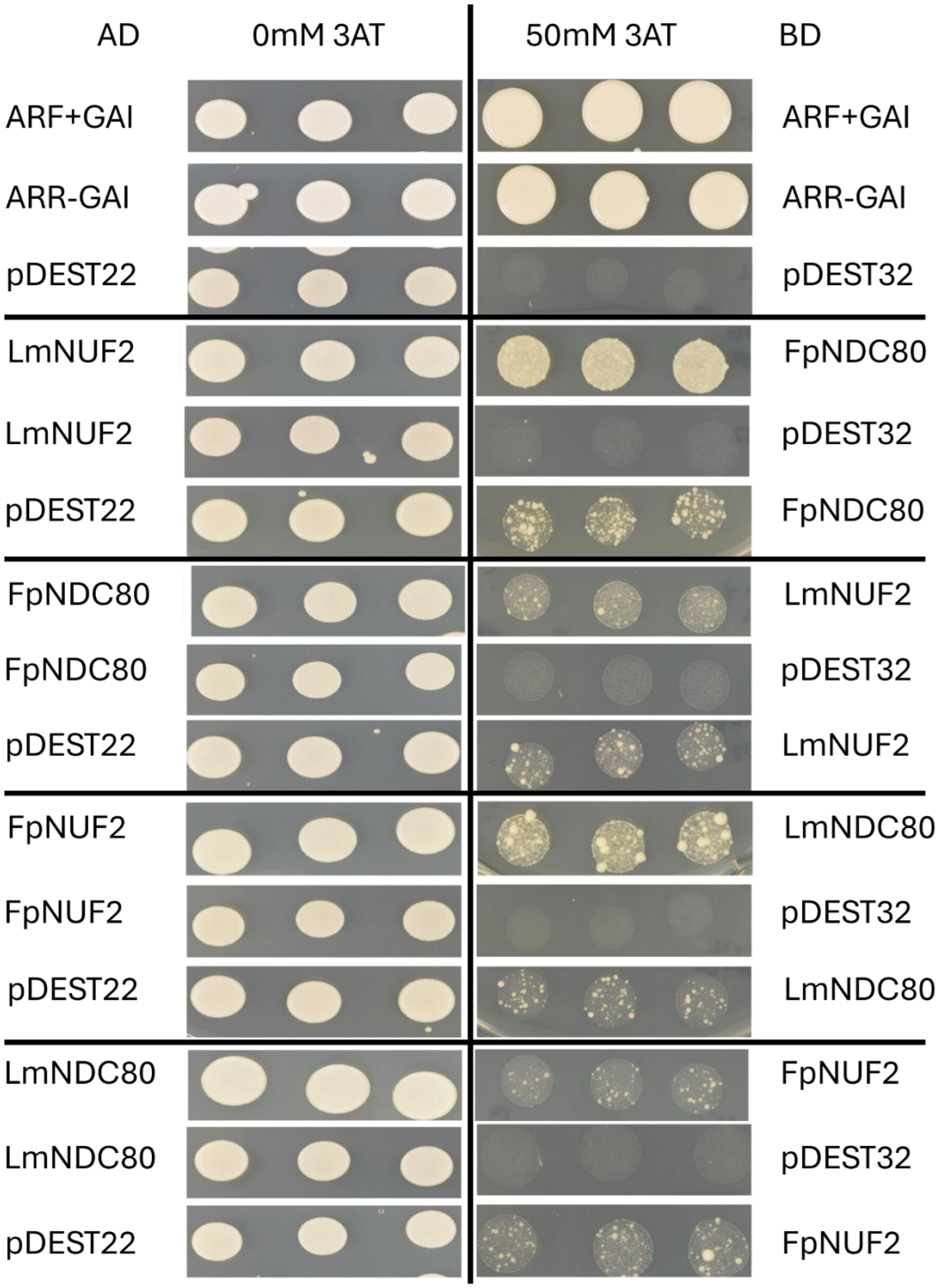

Figure 14: Y2H assay of interactions of homoeologs of NDC80 and NUF2 of *L. multiflorum* and *F. pratensis*. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).

5 Discussion

5.1 Knock-out lines of *A. thaliana*

5.1.1 *A. thaliana CENPC* knock-out lines

As mentioned in the results section, we received a sufficient amount of seeds from the transformed plants, but none of the selected plants carried a modification in the *CENPC* gene in the relevant guide sequence. This might indicate that CENPC protein is indispensable. As a part of the inner kinetochore, it plays a very important role during cell division (Wang and Dawe, 2017) and serves as a link between the DNA molecule itself, the CENH3 protein, and the outer kinetochore (Screpanti *et al*., 2011). Thus, the plant possibly needs two functional *CENPC* alleles to be viable.

Our hypothesis would also be confirmed by the fact that so far no study across eukaryotes has been able to obtain viable mutant lines that would carry a knock-out gene for *CENPC*. Research on the *CENPC* gene usually takes place on special cell lines and these are not constitutive knock-out lines, but lines with loss-of-function under certain conditions. An example can be the study of Fukagawa and Brown (1997), where they used the cell line DT40 (hyper-recombinogenic chicken B lymphocyte cell line), which carried a construct of the CENPC gene fused with a mouse steroid receptor, and when exposed to restrictive conditions, loss of function of the gene occurred. The same line was used in the study by Fukagawa *et al*. (2001) when the line displayed metaphase delay and chromosome missegregation but proceeded through the cell cycle until arrest at the G1 phase.

5.1.2 *A. thaliana NDC80* knock-out lines

In the case of *NDC80* gene, we managed to obtain several mutant lines carrying a knock-out allele of the gene using the CRISPR/Cas9 method. We think that it must be a heterozygous mutation, that is, one of the alleles must continue to remain in its original state (wild type). The reason for our consideration is the fact that the NDC80 protein is among the most essential kinetochore proteins and is involved in vital functions inside the cell, so its loss puts the plant at a major evolutionary disadvantage and the plant will expend increased resources to keep the gene functional (Cheeseman *et al*., 2006; Wei *et al*., 2007). This may also be related to our observation that in most lines, despite the changes in the nucleotide sequence, there was no disruption of the reading frame, Because the structure of the NDC80 protein is

highly conserved across organisms (Desai *et al*., 2003; Hori *et al*., 2003; McCleland *et al*., 2003; Meraldi *et al*., 2006).

This could also be related to the fact that the T2 generation of transgenic plants did not segregate as expected according to the Mendel's laws - in a 1:2:1 ratio. Taking into account the assumption that the homozygous mutation is lethal, it would be expected that 1/3 of the plants carry the wild-type genotype and 2/3 of the plants are heterozygous for the mutant allele. In the case of plants of the T2 generation originating from T1 line 11 (guide 1), there was a significant disruption of the segregation ratio. When I obtained only 2 positive plants from the 40 plants examined and the remaining number of plants carried the wild type genotype. The second mechanism, which may contribute to the disruption of the expected segregation of alleles in the T2 generation, was pointed out by studies of loss of function mutants in the *NDC80* gene, when in mutants of various species the chromosomes detach from spindle microtubules and inactivate the spindle checkpoint, resulting in extremely high rates of chromosome loss (Wigge and Kilmartin, 2001; Janke *et al.,*2001; Hori *et al.,* 2003; Martin-Lluesma *et al.,* 2002; McCleland *et al*., 2003; Gillet *et al.,* 2004). I think that this phenomenon could have partially manifested itself in our case as well, and if some chromosomes were lost, it was primarily chromosomes carrying a mutated allele of the *NDC80* gene, while chromosomes carrying a wild-type allele were favored by the plant.

5.2 Interactions of kinetochore proteins

The purpose of this work was to follow up on the conclusions of Majka *et al.* (2023) when in their research on genome dominance in the plant hybrid *Festulolium*, they concluded that the kinetochore proteins NNF1 and NDC80 are expressed in the plant only from the *Lolium* genome. NNF1 is part of the MIS12 complex and in the model plant *A. thaliana* has been shown to interact with MIS12 (Allipra *et al*., 2022). NDC80 is part of the NDC80 complex and interacts with NUF2 in *A. thalina* plants (Shin *et al*., 2018; Li *et al*., 2021). By studying the interactions between individual homoeologs of *Festuca* and *Lolium* proteins, we wanted to confirm or refute our hypothesis that the respective proteins NNF1 and NDC80 could play a role during the emergence of genome dominance in *Festulolium*.

However, the study of the assembly of kinetochores and other involved molecules faces several difficulties, given the complexity and importance of the entire mechanism inside

living organisms (Dos Santos *et al*., 2013). An appropriate way to investigate this is to focus on protein-protein interactions between individual molecules within the system (Galleta and Rusan, 2015). For our initial studies, we chose the Y2H method, which, according to Galleta and Rusan (2015), should be ideal for investigating kinetochore interactions. The main advantages are the simplicity of the experiments and the fact that the system is suitable for the investigation of multiprotein complexes. The reaction itself takes place inside the yeast nucleus. However, the Y2H system faces several disadvantages. Specifically, it is the number of false positives and negatives (Galleta and Rusan, 2015).

In our case, we observed the occurrence of false positives. All studied proteins placed in the yeast expression plasmids were able to activate the transcription of the *HIS3* gene without the appropriate binding partner (Galleta and Rusan, 2015). According to the study by Serebriiskii and Golemis (2001), proteins that are in the bait vector (pDEST32) are more prone to false positives, which was also evident in our case, when 6 out of 8 cases of false positives were in the bait vector. To prevent the occurrence of false positives and improve the results, 3-AT can be added to the selection medium. 3-AT serves as a competitive inhibitor of the *HIS3* gene and only yeast colonies with the strong bond between the interacting partners can survive on the selection media (Durfee *et al*., 1993). However, we tried this option, but it did not bring any improvement. Another option is to use multiple types of reporter genes and/or multiple types of promoters under which the reporter genes are located (Rajagopala and Uetz, 2009). According to previous studies, it is advantageous to divide the respective proteins into smaller fragments and not examine the proteins as a whole. Especially if the functional domains of the respective proteins are known (Flajolet *et al*., 2000; Formstecher *et al.*, 2005; Boxem *et al*., 2008).

The obtained results also showed that FpMIS12 and FpNNF1 proteins do not interact with each other. Available literature suggests that MIS12 and NNF1 proteins should interact across organisms (Li and Dawe, 2009; Musacchio and Desai, 2017; Allipra *et al*., 2022) and it is therefore very unlikely that these two proteins would not interact with each other in this assay. Therefore, in my opinion, the protein pair FpMIS12 and FpNNF1 was a false negative. The fact that the proteins did not interact could be explained by an error in Y2H. It has been shown that in some cases, the respective AD or BD domains attached to the investigated proteins can be put into a wrong configuration during the interaction and this prevents the proper

binding of the two domains. Furthermore, we must take into account the fact that foreign proteins are formed in the yeast organism. For their proper function, some proteins need to undergo certain post-translational modifications, which yeast may not be capable of (Rajagopala and Uetz, 2009; Bruckner *et al.,* 2009).

The last possibility to avoid the problems mentioned above is to use another method to study protein-protein interactions. Today, the most widely used methods are in-planta assays based on fluorescence measurements. Examples can be BiFC (Bimolecular fluorescence complementation), FRET (Fluorescence Resonance Energy Transfer), FIDA (Flow-induced dispersion analysis), FLIM (Fluorescence-lifetime imaging microscopy) and others (Ujlaky-Nagy *et al*., 2017; Pedersen *et al*., 2019; Chen *et al*., 2024; Bonilla and Shrestha, 2024).

6 Conclusion

In the theoretical part, the diploma thesis dealt with the issue of polyploidy with greater emphasis on interspecific hybridization, i.e. crossing of two individuals of different species. The resulting hybrids therefore carry two different genomes originating from individual parents. The second part concerned the phenomenon known as non-mendelian inheritance. Under this general term, we can include a whole range of mechanisms or phenomena in which the fundamental laws of Mendelian inheritance are violated during the inheritance of some traits. It can lead to genome dominance, mentioned more in the theory section, which is also closely connected with interspecific hybrids. Genome dominance manifests itself in the fact that one of the parental genomes within the hybrid becomes more dominant over the other and parts of the submissive genome are gradually eliminated. The last part focused on kinetochore proteins and their potential role during the genome dominance in the hybrid *Festulolium* species, as pointed out in Majka *et al*. (2023).

The practical part had two main goals. The first task was to obtain mutant lines of *A. thaliana* in certain kinetochore genes, namely *CENPC* and *NDC80.* In the case of the *NDC80* and *CENPC* genes, it was a line that would carry a knock-out allele of the respective genes, and the CRISPR/Cas9 method was used to create the lines. For the *CENPC* gene, we were unable to obtain any plants carrying a modified allele of the gene, most likely this is due to the fact that the *CENPC* gene is vital for the survival and proper functioning of the organism. For the *NDC80* gene, in the T1 generation, I obtained 9 plants that carried the gene modification in the proposed guide 1 sequence and 8 plants that had the altered gene in the guide 6 sequence. In the T2 generation, I subsequently obtained 2 promising lines that carry a heterozygous change in the gene allele *NDC80* in the respective guide 1 sequence and are also negative for the presence of an active cassette for CRISPR/Cas9. The work done and the knowledge gained in this diploma thesis laid the foundations for ongoing research on the aforementioned topics.

The second goal was to study protein-protein interactions between two pairs of kinetochore proteins, MIS12 and NNF1 and NDC80 and NUF2, from the plants *F. pratensis* and *L. multiflorum* and evaluate whether there are any observed differences in the strength of interactions between proteins originating from the same organism and interactions

between proteins from different organisms, i.e. one protein originated from *F. pratensis* and the other from *L. multiflorum*. Due to the aforementioned difficulties, the results in this part are inconclusive and it is necessary to perform further optimization of the Y2H method or choose another method to study protein-protein interactions.

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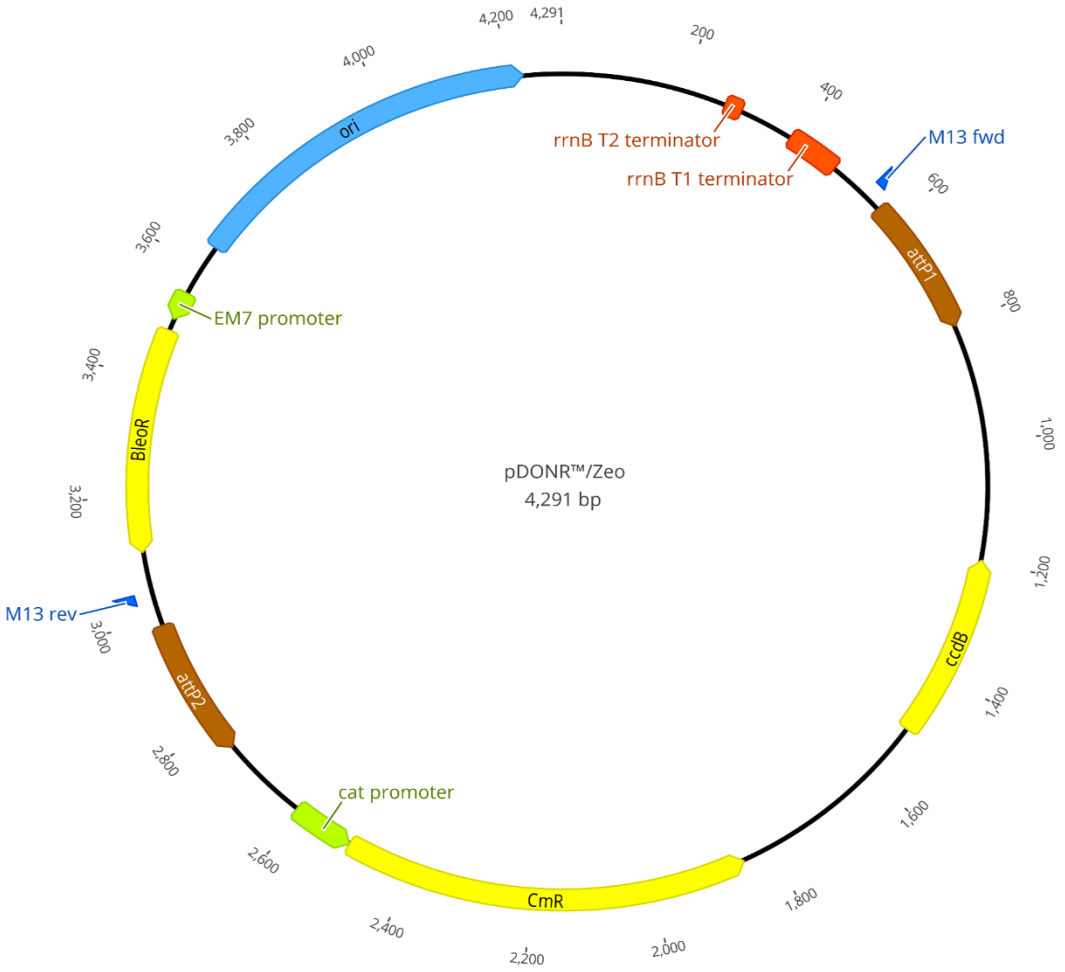
8 List of abbreviations

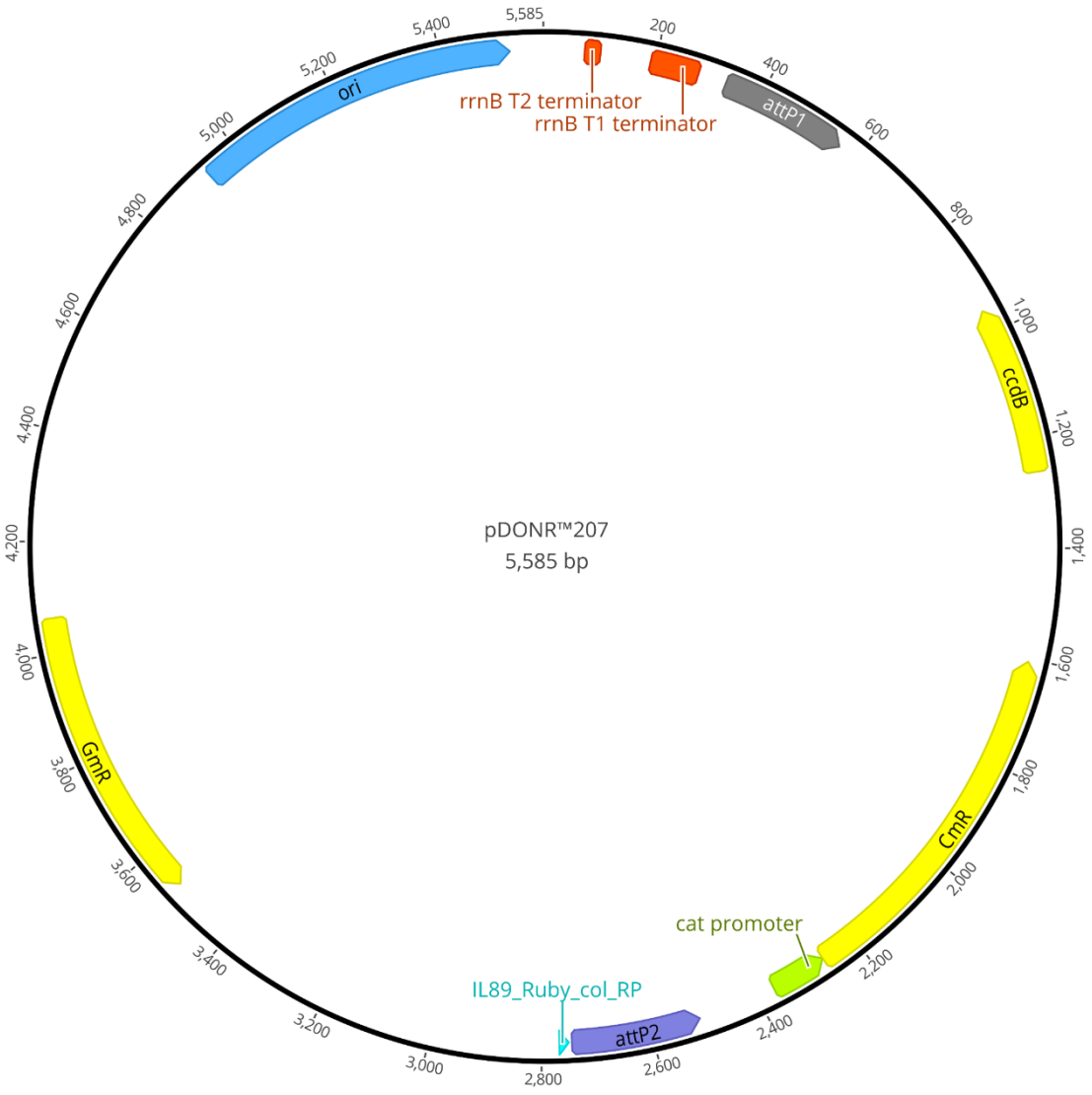
µl	microliter
µM	micromol
½ MS	1/2 Murashige and Skoog medium
3AT	3-amino-1,2,4-triazole
Å	Ångström
Acetosyringone	4'-Hydroxy-3',5'-dimethoxyacetophenone
AD	Activation domain
ARF	Auxin-responsive transcription factor
ARR	Arabidopsis response regulator
AtNNF1	a protein that comes from the *A. thaliana* plant
BD	DNA-binding domain
BiFC	Bimolecular fluorescence complementation
CCAN	constitutive centromere associated network
cDNA	Complementary DNA
CENH3	Centromere-specific histone H3
CENP-A	Centromere Protein A
CENP-C	Centromere Protein C
CENP-O	Centromere Protein O
CENP-S	Centromere Protein S
CENP-X	Centromere Protein X
cm	centimeter
Col-0	*Arabidopsis thaliana* ecotype: Columbia 0
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
DNA	Deoxyribonucleic acid
DSN1	MIND kinetochore complex component
FIDA	Flow-induced dispersion analysis
FLIM	Fluorescence-lifetime imaging microscopy
FpNNF1	a protein that comes from the *F. pratensis* plant
FRET	Fluorescence Resonance Energy Transfer
g	Gravity force
GAI	gibberellic acid insensitive
HIS3	Gene for imidazoleglycerol-phosphate dehydratase HIS3
kb	kilobase
KMN	network composed of KNL1 complex, MIS12 complex and NDC80 complex
KNL1	kinetochore null 1
LB	Lysogeny broth
LiAc	lithium acetate
LmNNF1	a protein that comes from *L. multiflorum* plant
Mif2	A yeast homologue of CENPC
min	minute
min/kb	minute per kilo
MIS12	minichromosome instability 12
ml	milliliter

mM	milimol
mRNA	messenger RNA
MUN1	meristem unstructured-1
NDC80	nuclear division cycle 80
ng	nanogram
ng/µl	nanogram per microliter
NMR	nuclear magnetic resonance
NNF1	necessary for nuclear function 1
NUF2	nuclear filament-containing protein 2
ºC	degrees Celsius
OD600	optical density measured at 600nm
PCR	polymerase chain reaction
PEG 3350	Polyethylene Glycol 3350
Ph1	*Pairing homoeologous 1* gene
pmol	picomole
PPIs	protein-protein interactions
RNA	Ribonucleic acid
Rpm	revolutions per minute
SAC	spindle assembly checkpoint
SD	Synthetic define medium
SPC24	spindle pole body component 24
SPC25	spindle pole body component 25
T1	Generation of plants derived from directly transformed seeds
T2	Generation of plants derived from seeds of T1 generation plants
TE buffer	Tris-EDTA buffer
TEs	transposable elements
V	volt
Y2H	Yeast two-hybrid
YEB	Yeast extract beef broth
YPD	Yeast extract–peptone–dextrose medium

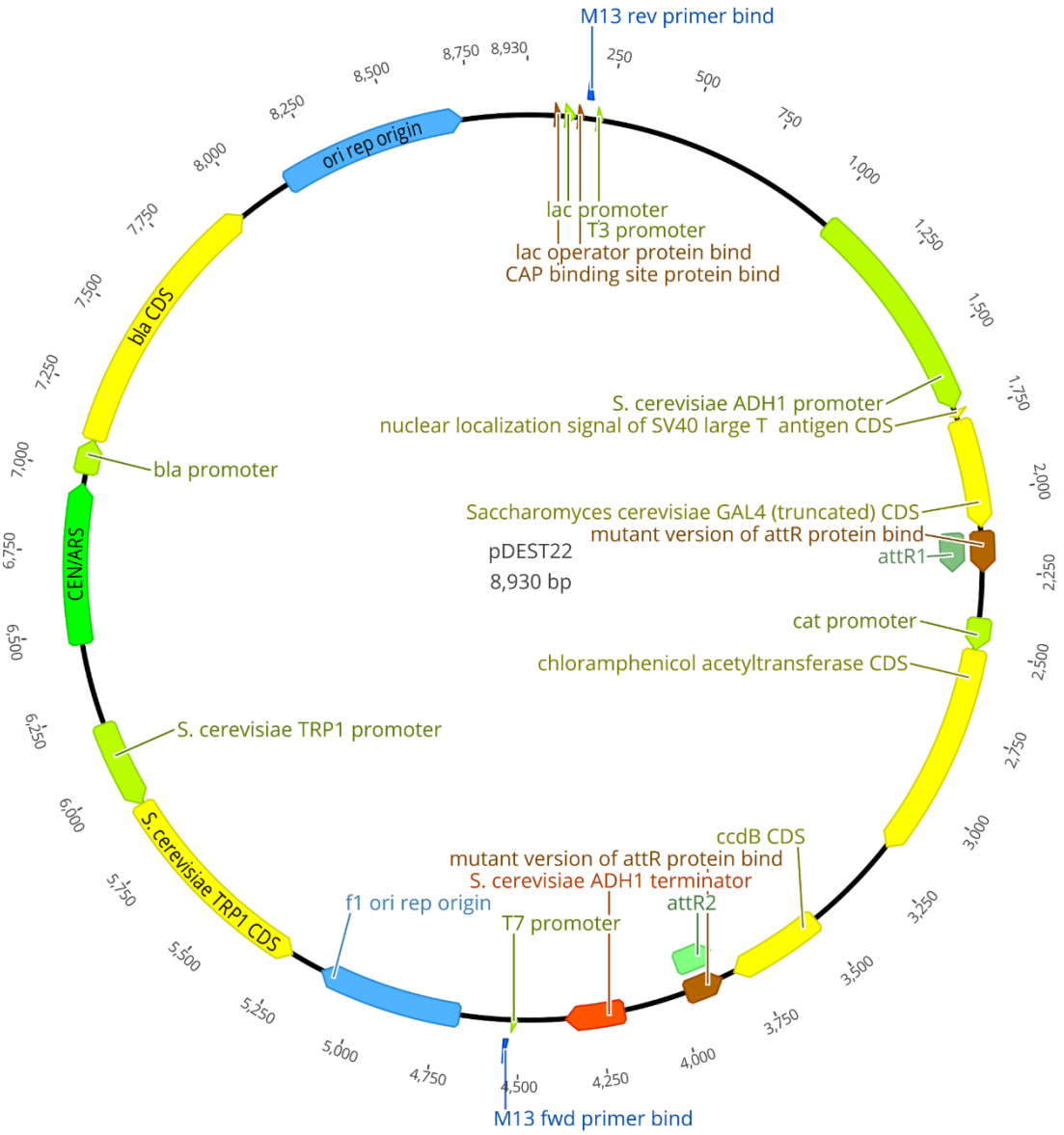
9 Appendices



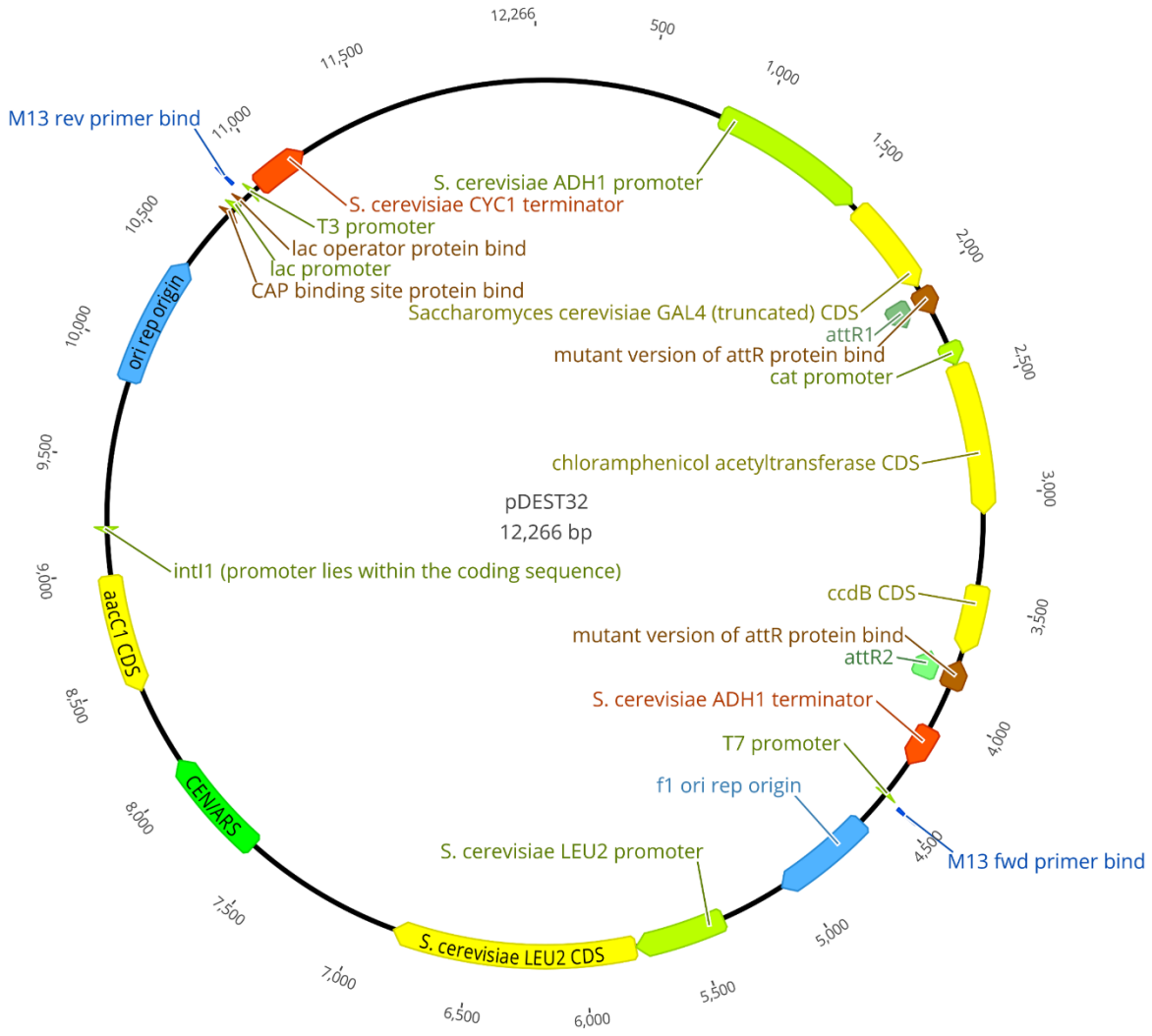
Appendix 1: Vector map of pDONR/Zeo; *BleoR* = gene fo Zeocin resistance; *ccdB* = "killer" gene; *ori* = origin of replication; *CmR* = gene for Chloramphenicol resistance.



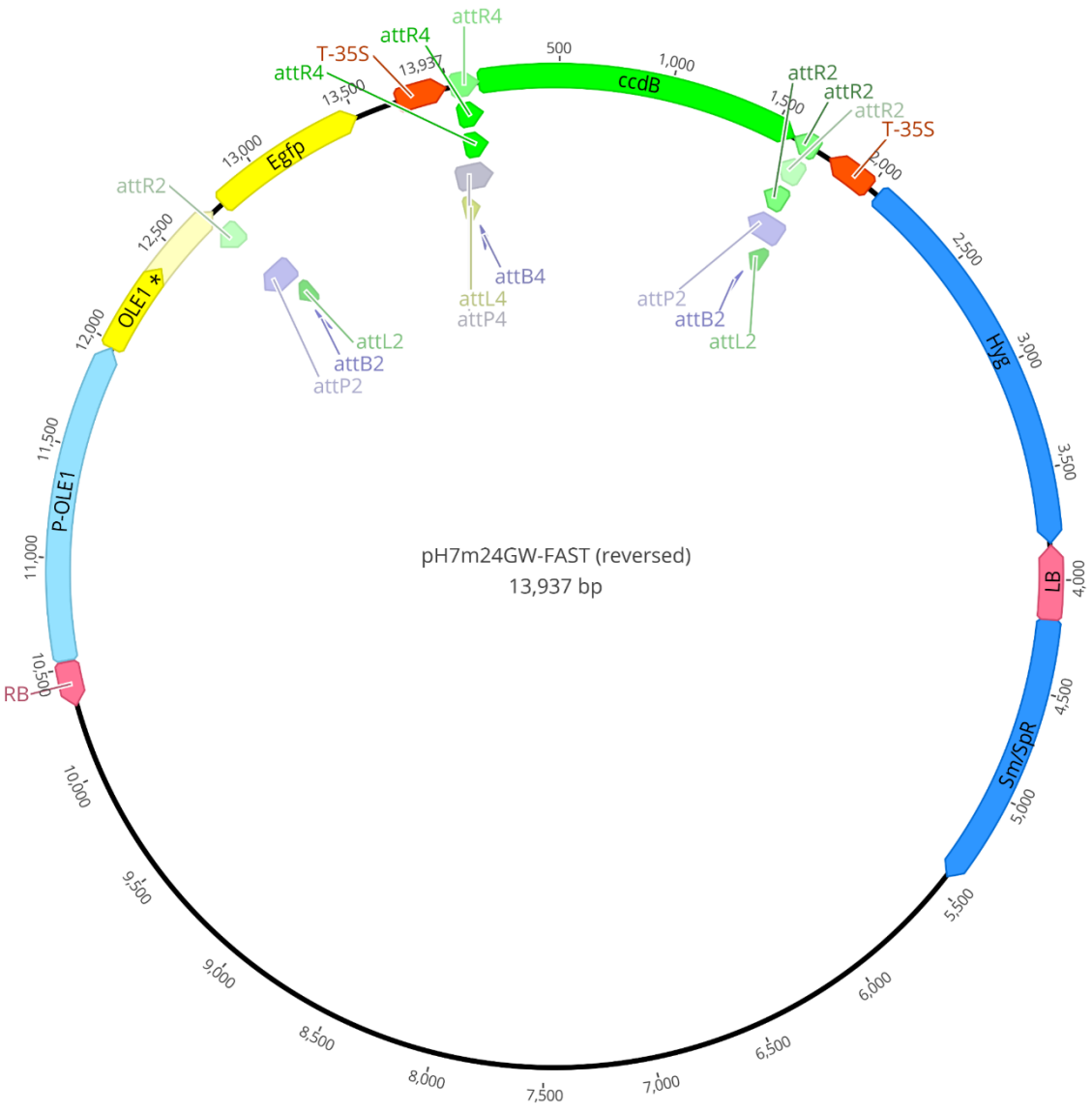
Appendix 2: Vector map of pDONR/207, *CmR* = gene for chloramphenicol resistance; *ccdB* = "killer" gene; *ori* = origin of replication; *GmR* = gene for gentamycin resistance.



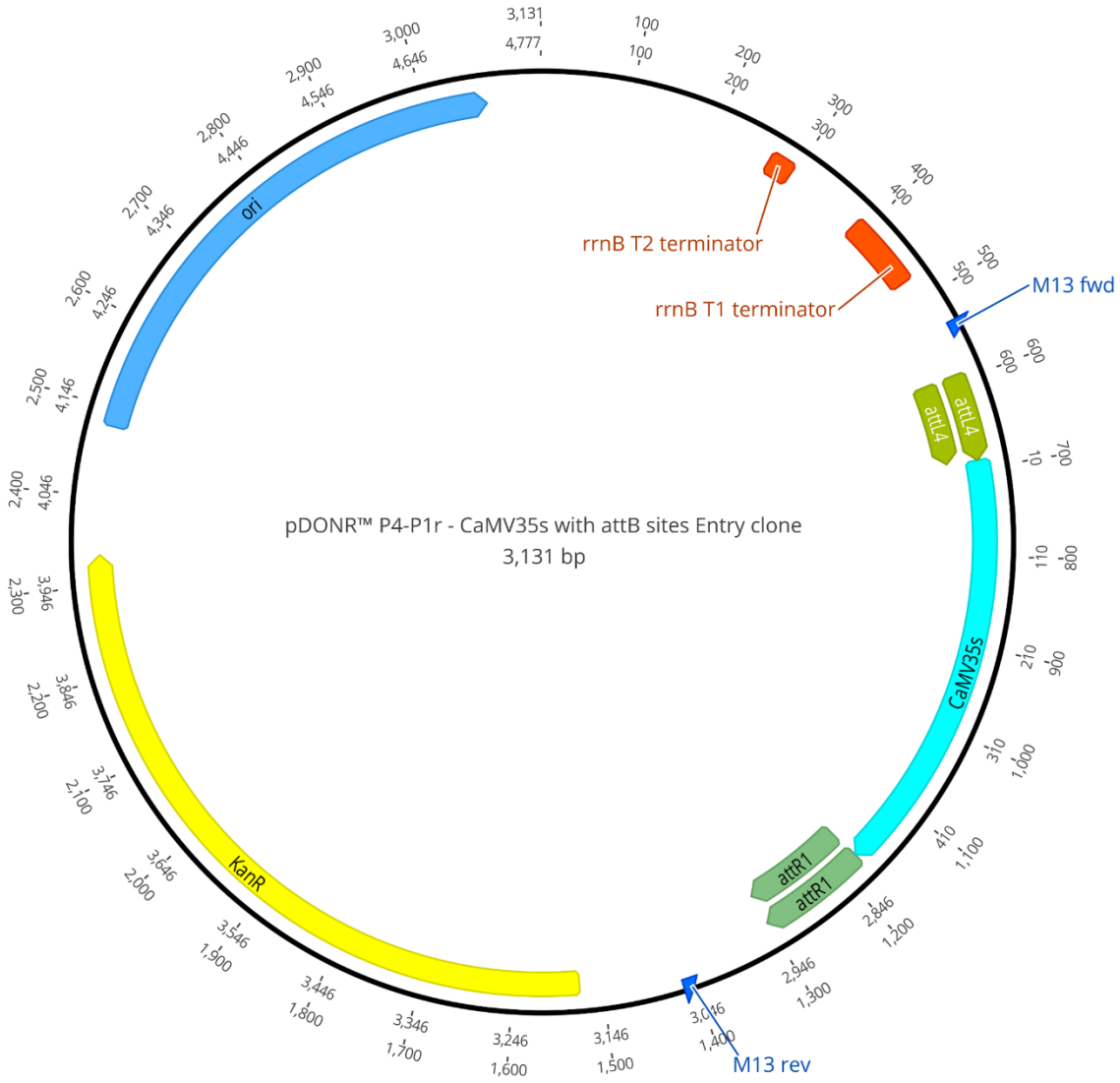
Appendix 3: Vector map of pDEST22; *bla* = gene for ampicillin resistance; *GAL4* = gene for GAL4 activation domain; *TRP1* = gene for phosphoribosyl-anthranilate isomerase; *ccdB* = "killer" gene.



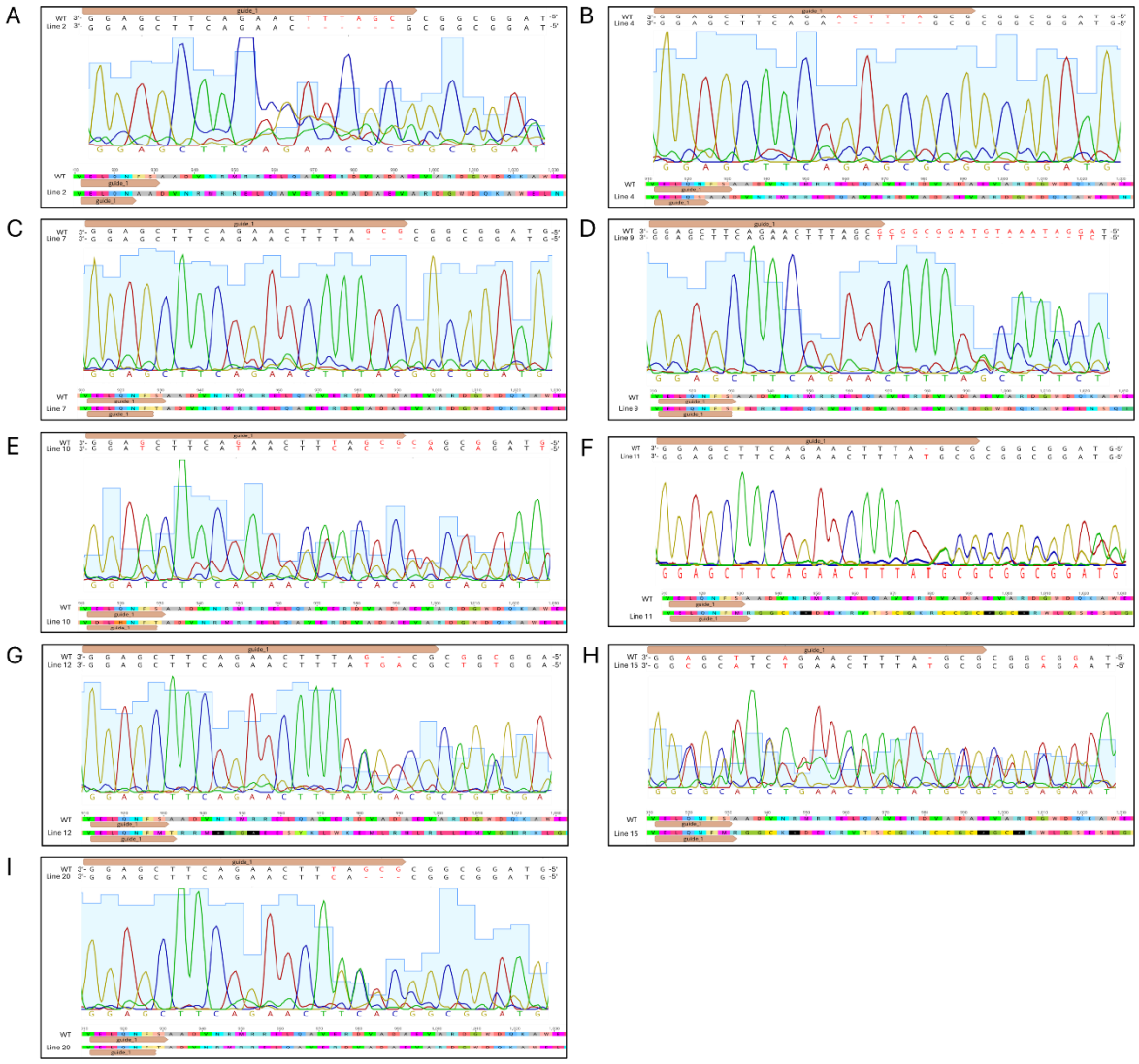
Appendix 4: Vector map of pDEST32; *GAL4* = gene for GAL4 DNA-binding domain; *LEU2* = gene for beta-isopropylmalate dehydrogenase; *ccdB* = "killer" gene; *aacC1* = gene of gentamicin resistance.



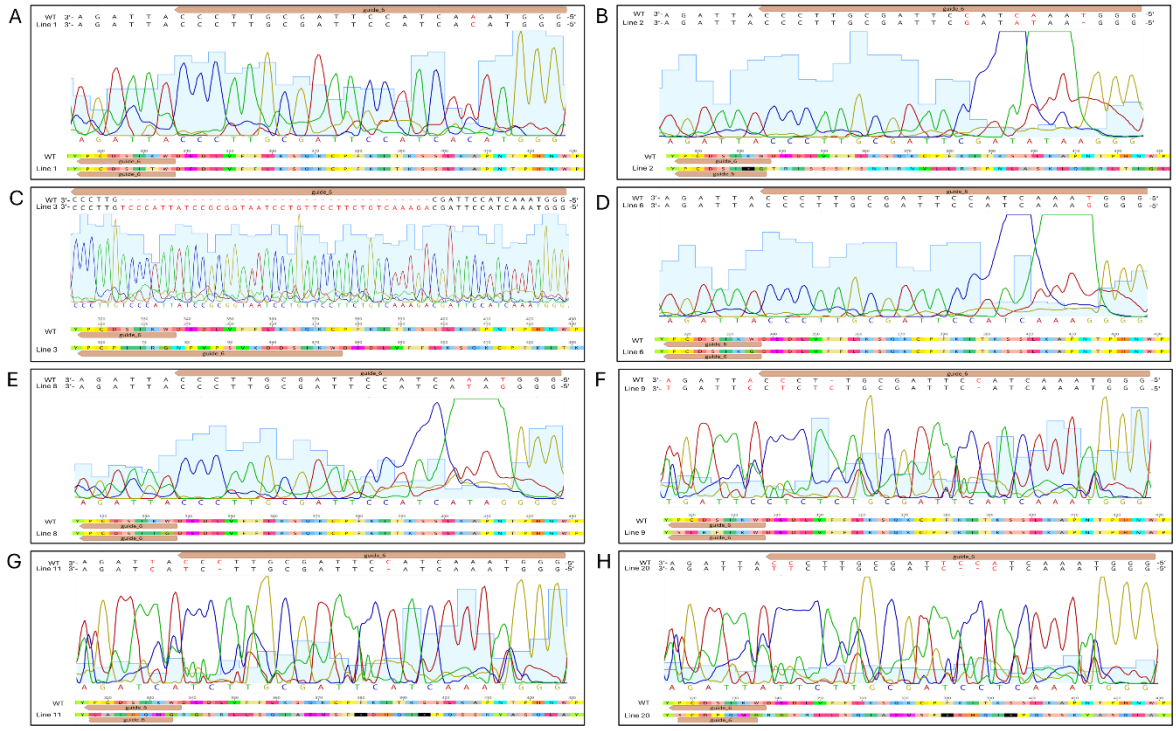
Appendix 5: Vector map of pH7m24GW-FAST; *Hyg* = gene for hygromycin resistance, *EGFP* = gene for green fluorescent protein; *SpR* = spectinomycin resistance; *ccdB* = "killer" gene.



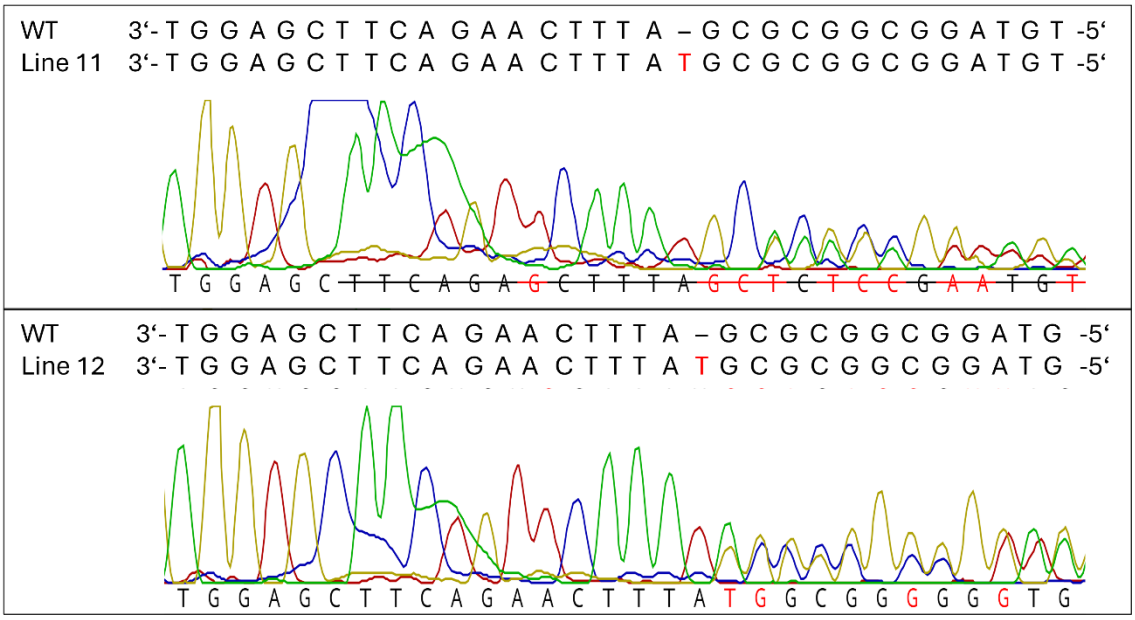
Appendix 6: Vector map of pDONR P4-P1r with CaMV35s promotor; *KanR* = gene kanamycin resistance; *ori* = origin of replication.



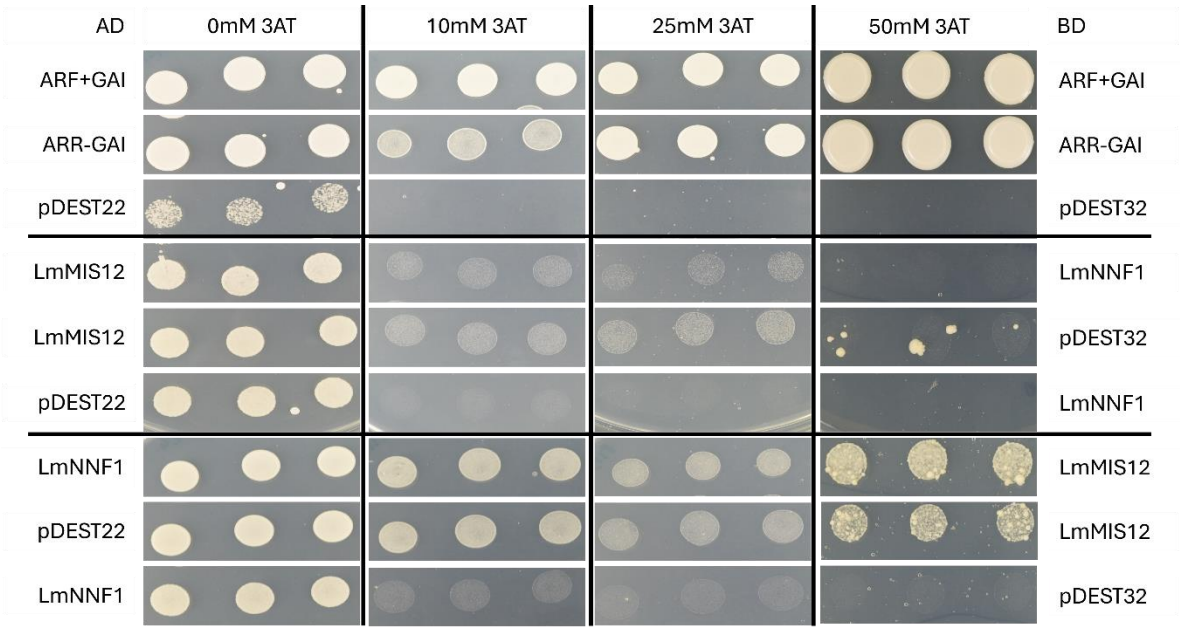
Appendix 7: Detailed Sanger sequencing results of T1 guide 1 lines and their amino acid sequence, (A) Line 2, (B) Line 4, (C) Line 7, (D) Line 9, (E) Line 10, (F) Line 11, (G) Line 12, (H) Line 15, (I) Line 20



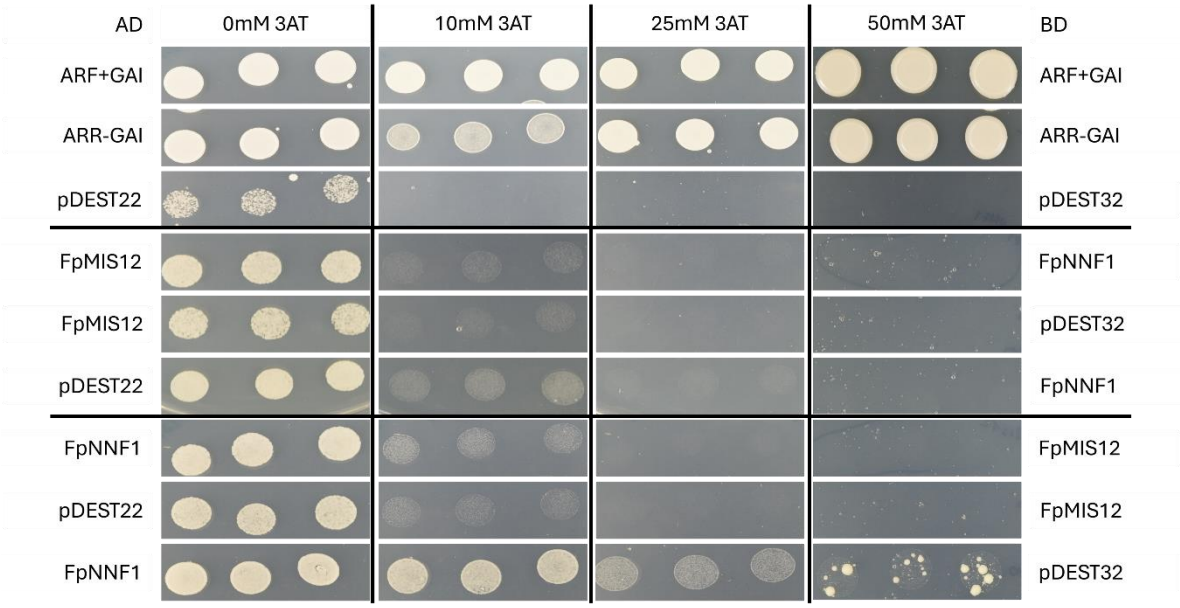
Appendix 8: Appendix 8: Detailed Sanger sequencing results of T1 guide 6 lines and their amino acid sequence, (A) Line 1, (B) Line 2, (C) Line 3, (D) Line 6, (E) Line 8, (F) Line 9, (G) Line 11, (H) Line 20.



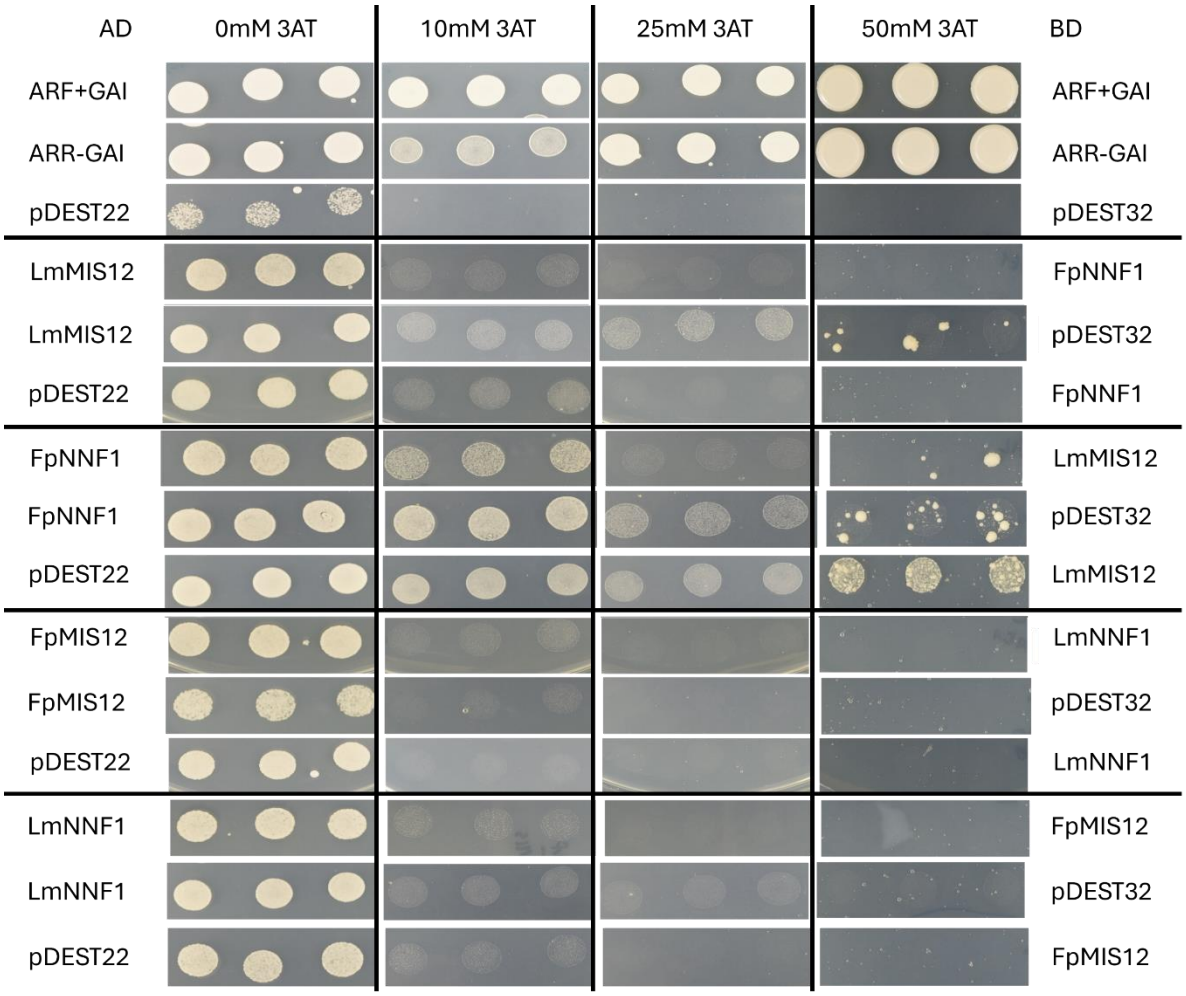
Appendix 9: Results of Sanger sequencing of T2 lines (from parental T1 line 11; guide 1). (A) Line 11, (B) Line 12.



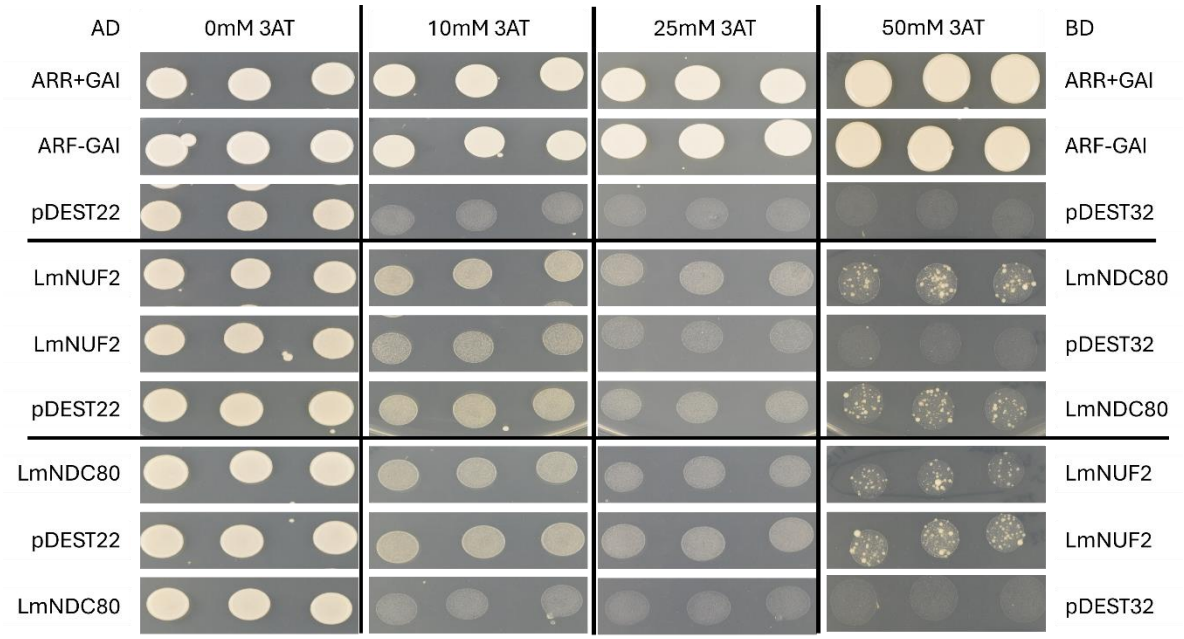
Appendix 10: Y2H assay of interactions of *L. multiflorum* proteins LmMIS12 and LmNNF1 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to a final concentration of 10mM (3AT = 3-amino-1,2,4-triazole); 25mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 25mM; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 50mM.



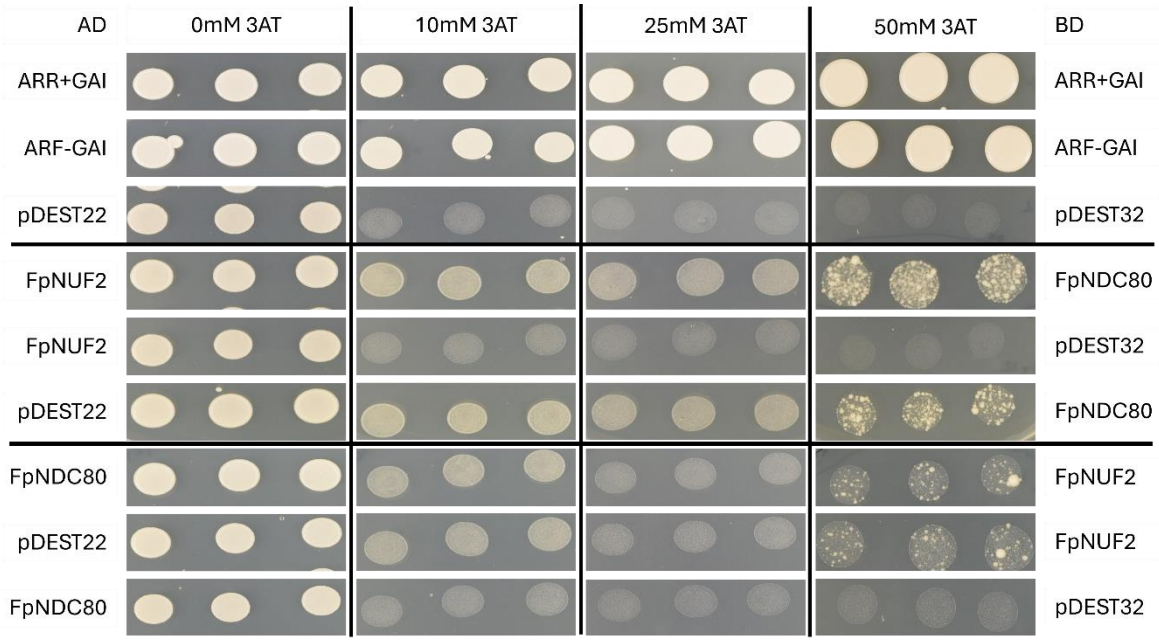
Appendix 11: Y2H assay of interactions of *F. pratensis* proteins FpMIS12 and FpNNF1 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to a final concentration of 10mM (3AT = 3-amino-1,2,4-triazole); 25mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 25mM; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 50mM.



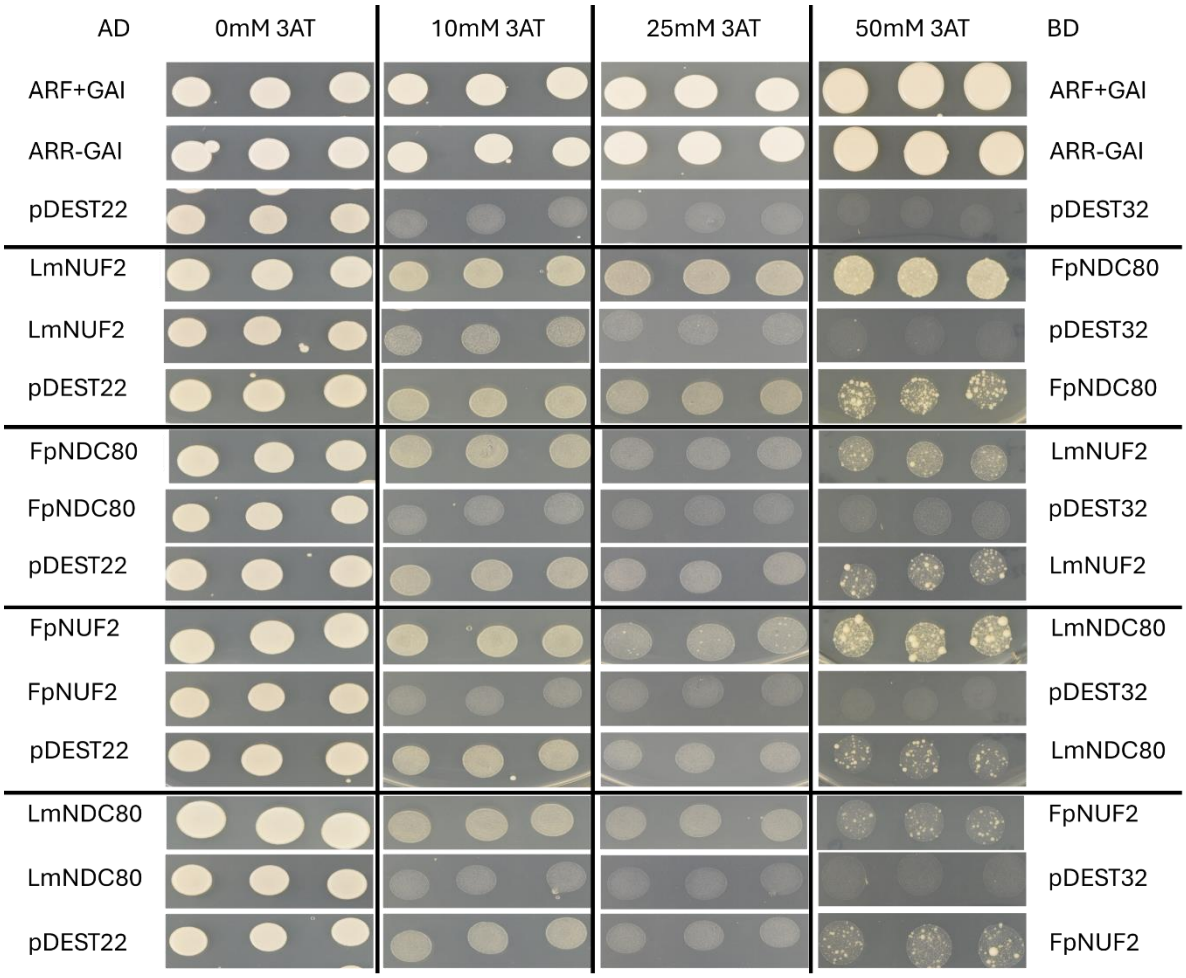
Appendix 12: Y2H assay of interactions of homoeologs of MIS12 and NNF1 of *L. multiflorum* and *F. pratensis*. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to a final concentration of 10mM (3AT = 3-amino-1,2,4-triazole), 25mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 25mM; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with addition of 3AT to final concentration of 50mM.



Appendix 13: Y2H assay of interactions of *L. multiflorum* proteins LmNDC80 and LmNUF2 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to a final concentration of 10mM (3AT = 3-amino-1,2,4-triazole), 25mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 25mM; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).



Appendix 14: Y2H assay of interactions of *F. pratensis* proteins FpNDC80 and FpNUF2 and levels of their auto-activation. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to a final concentration of 10mM (3AT = 3-amino-1,2,4-triazole), 25mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 25mM; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).



Appendix 15: Y2H assay of interactions of homoeologs of NDC80 and NUF2 of *L. multiflorum* and *F. pratensis*. In three technical repetitions. AD = activation domain (prey vector); BD = DNA-binding domain (bait vector); ARF+GAI = positive control (ARF = auxin-responsive transcription factor; GAI = gibberellic acid insensitive); ARR-GAI = positive control (ARR = Arabidopsis response regulator); pDEST22+pDEST32 = negative control; 0mM 3AT = SD medium without Histidine, Leucine, Tryptophan; 10mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to a final concentration of 10mM (3AT = 3-amino-1,2,4-triazole), 25mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 25mM; 50mM 3AT = SD medium without Histidine, Leucine, Tryptophan with the addition of 3AT to final concentration of 10mM (3AT = 3-amino-1,2,4-triazole).