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Bachelor thesis

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Cytonuclear interactions in grass autopolyploids and allopolyploids

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Summary:

The aim of this bachelor thesis was to evaluate intracellular changes following polyploidization and to count copy numbers of genes involved in cytonuclear interactions in auto- and allopolyploids. The leaf mesophyll cells from different plants were observed using a confocal microscope and evaluated in IMARIS software. I estimated cell and nuclear volumes, and numbers and volumes of chloroplasts. My results revealed that doubling the nuclear genome is accompanied by the increase in the nuclear volume by about 56% and cell volume by 21%. This is further pronounced in successive generations up to about 70% and 30%, respectively. Moreover, the obtained data revealed that the strategy to mitigate the imbalance in the stoichiometry of cytonuclear interactions in polyploids involve increase in the number (by about 15%) and volume (by about 17%) of chloroplasts, former being increased in the successive generations (to about 30%), while latter being purified (to about 7%). Interestingly, heterosis was not revealed for almost any parameters in homoploids and allopolyploids.

Subsequently, the absolute copy number of the genes for the selected set of genes involved in cytonuclear interactions should was to be determined by the ddPCR method. This part could not be performed due to technical problems.

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Cílem této bakalářské práce bylo vyhodnocení intracelulárních změn následujících polyploidizaci a spočítat kopie genů zapojených do cytonukleárních interakcí u auto- a allopolyploidů. Nejprve byla provedena literární rešerše o polyploidii a cytonukleárních interakcích. Poté bylo pomocí konfokálního mikroskopu a softwaru IMARIS provedeno pozorování buněk a vyhodnocení jejich rozměrů, velikostí jádra a počtu a velikosti chloroplastů. Mé výsledky prokázaly, že je zdvojení genomu doprovázeno zvýšením objemu jádra o asi 56 % a objemu buněk o 21 %. To je dále patrné v po sobě jdoucích generacích až o 70 % a 30 %. Získaná data navíc odhalila, že strategie ke zmírnění nerovnováhy ve stechiometrii cytonukleárních interakcí u polyploidů zahrnuje zvýšení počtu (asi o 15 %) a objemu (asi o 17 %) chloroplastů, přičemž dříve se zvyšuje v následujících generacích (na přibližně 30 %), zatímco druhý je purifikován (na přibližně 7 %). Zajímavé je, že u homoploidů a allopolyploidů nebyla heteróza odhalena téměř u žádných parametrů.

Následně měl být metodou ddPCR stanoven absolutní počet kopií genů pro vybraný soubor genů účastnících se cytonukleárních interakcí. Tuto část nebylo možné provést z důvodu technických problémů.

Statement

I declare that I have prepared this bachelor's thesis independently using the above-mentioned literary sources and under the guidance supervision of RNDr. David Kopecký, PhD.

In Olomouc…… ……

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I would like to thank my supervisor RNDr. David Kopecký PhD. and his team for their dedicated time, willingness, patience, and professional guidance in drafting the bachelor's thesis. I would also like to thank all the staff of the Center for Structural and Functional Plant Genomics of the Institute of Experimental Botany of the Czech Academy of Sciences in Olomouc.

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1 Introduction

Polyploidization is generally defined as the acquisition of more than two chromosome sets. Polyploidy is nowadays recognized as an important force of plant evolution (Ramsey and Schemske 1998); it was discovered more than century ago (Strasburger, 1910) and it naturally occurs in many eucaryotic taxa. Whole genome duplication (WGD) is the basis for many major episodes ofdiversification in land plants (Van de Peer et al., 2017) and remains a prominent process in plant speciation (Barker et al., 2016).

There are three distinct compartments in plant cell, where DNA is stored: the nucleus, mitochondria and plastids. Therefore, newly developed polyploids stand before a challenge of facing the disruption of well-tuned stoichiometry between the nuclear-encoded and organelle-encoded genes involved in cytonuclear interactions (such as RubisCo, whose large subunit is encoded by nuclear gene *rbcL* and small subunit is encoded by chloroplast gene *rbcS*), as doubling the copy numbers of nuclear genes after whole genome duplication is not accompanied with the duplication of the number of organelles (Sharbrough et al., 2017). One indication that polyploidy has significant stoichiometric implications for cytonuclear interactions is that organelle-targeted genes appear to be one of the first and most common classes of nuclear genes to return to single copy following a WGD. In addition, the well-known positive correlation between nuclear genome size and cell size (Beaulieu et al., 2008) suggests that plants may be able to store more organelles per cell as nuclear genome copy number and cell size increases, for example the alfalfa polyploids exhibit elevated chloroplast number per cell relative to diploids (Bingham, 1968).

Major challenge can occur in allopolyploids and interspecific hybrids for genes involved in cytonuclear interactions. The typical inheritance of the cytoplasmic genomes is uniparental (usually maternal), while the nuclear-encoded genes come from both parents and these genes may be diversed between parental genomes and non-interchangeable (Sharbrough et al., 2017). The cytonuclear interaction has been the focus of interest of plant scientists for almost two decades and various databases depicting genes involved in the cytonuclear interactions have been released. Most recently, the CyMIRA database, as a Cytonuclear Molecular Interactions Reference for Arabidopsis including 910 nuclear-encoded genes involved in direct cytonuclear molecular interactions (i.e., components of cytonuclear enzyme complexes) was developed (Forsythe et al., 2019). These databases will allow further studies to uncover biological mechanisms that contribute to the reestablishment of the stoichiometry between cytoplasmic and nuclear genes after polyploidization. Such studies will extend our knowledge about the evolutionary relevance of polyploidization, as one of the main mechanisms of plant speciation.

My Bc. thesis is focused on the changes associated with cytonuclear interactions in newly and well-established auto- and allopolyploids (*Lolium*, *Festuca* and their hybrids).

2 The work goal

1. A literature search on the topic of polyploidy and cytonuclear interactions in polyploids.
2. Microscopic analyses of plant cells in diploid and polyploid grasses (*Lolium multiflorum* and *Festuca pratensis*) and their hybrids including size and number of chloroplasts.
3. Determination of the absolute gene copy number for the selected set of genes involved in cytonuclear interactions using ddPCR.

3 Literary review

Polyploidy is defined as a state, when the organism consists of three or more sets of chromosomes. These multiplied sets of chromosomes coexist in one nucleus and can be stably inherited into the progenies. Many of these polyploid species are well adapted to their environments. In fact, recent findings in genome research indicate that many species that are currently diploid, including humans and model plant Arabidopsis, were derived from their polyploid ancestors (Van de Peer & Meyer, 2005).

However, polyploidization event causes dramatic changes in the genome structure and nuclear organization. It introduces several major challenges on cell cycle processes (e.g., mitoses, meiosis), cell physiology (e.g., metabolism, growth, stoichiometry), regulation of gene expression and genome stability. This might be further exacerbated by the presence of multiple chromosome sets originating from different species. Interspecific hybridization is frequently accompanied with whole genome doubling (polyploidization) and allopolyploids (polyploids with diverged chromosome sets) are frequent in plants. However, genetic and epigenetic changes associated with allopolyploidization can lead to the instability of hybrid genome. Thus, hybrid genomes are frequently stabilized in successive generations via a process called diploidization. It is a gradual transformation of polyploidy into diploid state with the complete or partial loss of duplicated genes. Species, which underwent polyploidy event(s) and subsequent diploidization are called paleopolyploids. On contrary, neopolyploids still possess multiple sets of chromosomes (Zhang et al., 2019).

Polyploidy is common among plants, while being rare in animals. In plant kingdom, we can observe high tolerance to polyploidization, and it is assumed that the estimation of polyploidy incidence is widely variable, ranging from 30 % (Stebbins 1971) to 70 % (Masterson 1994) in angiosperms. In animals, there are only limited number of polyploid species, such as some frogs (Shmid et al. 2015), fish (Zhou and Gui, 2017), insects (Li et al., 2018), and mammals (Acharya and Ghosh, 2016). Interestingly, polyploid tissues can be found in otherwise diploid organisms, including hepatocytes in a human liver.

3.1 Evolutionary force of polyploidization

Polyploidization is considered as one of the major drives of plant species diversification and plays an important role in the development of plant genomes. Polyploidy leads to the presence of multi-copies of genes, which coexist. Functional development of the duplicated genes may provide new function of the accessorial copies (neo-functionalization), which may further promote genome adaptation and plasticity of plant species (Ohno, 1970; Cheng et al., 2018). Researching these phenomena can improve our understanding of the genetic mechanisms underlying gene development, and plant speciation and domestication. Polyploidization is considered a mode of speciation with far-reaching ecological and evolutionary consequences. In fact, polyploid organisms and their populations can better

tolerate extreme environments compare to the diploid progenitors due to their increased genetic variation. However, persistence and diversification of polyploid lineages depends on their fitness and the long-term evolutionary consequences of polyploidization. In some cases, newly formed polyploids can have reduced ability to survive and/or reproduce. These organisms can represent evolutionary dead end.

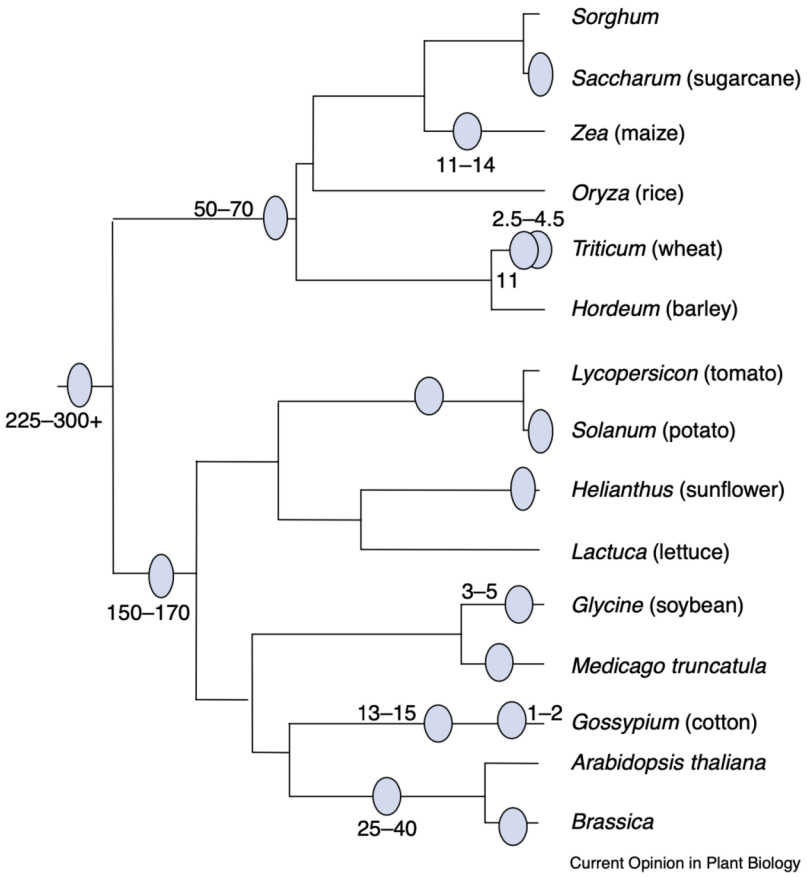

Figure 1: Inferred polyploidy events during the evolution of angiosperms, where the blue shaded ovals are indicating suspected large-scale duplication events. (Adopted from Adams and Wendel, 2005)

3.2 Types of polyploids

3.2.1 Autopolyploids

Autopolyploids are polyploids with extra chromosome sets derived from a single species. Autopolyploids can arise from a genome doubling, either spontaneous or induced. For a long time, autopolyploidy was considered rare and representing evolutionary dead end due to the meiotic problems (formation of multivalents and consequent improper segegation) leading to the reduced fertility (Stuessy and Schneewiess, 2019, Clausen et al., 1945; Stebbins, 1971). The frequency of autopolyploidy among plants can be only debated, because many autopolyploids have escaped recognition due to their morphological similarities to the diploid progenitors and being concealed among common diploid taxa (Soltis et al., 2007). Interestingly, autopolyploids are widely utilized in crop production and many crops are autopolyploids including potato, some banana species, watermelon, and sugarcane.

3.2.2 Allopolyploids

Allopolyploids are polyploids with chromosome sets derived from different species, which are more or less related. Two (or more) divergent genomes in allopolyploids vary in chromosomal homology, based on the phylogenetic relationship of the parental species. In the case of hybridization between distantly related species, chromosomal homology can be low enough to hamper homoeologous chromosome pairing during meiosis (Svačina et al., 2020). Conversely, allopolyploids that originated from the cross between closely related species carry chromosomes with a much higher degree of homology. Therefore, their homologous chromosomes have potential to pair and recombine during meiosis (Ramsey and Schemske, 1998; Sun et al., 2017). Interspecific hybridization is accepted as a key evolutionary force for adaptation and speciation in plant groups (Barton 2001). Typical example of allopolyploid is bread wheat *Triticum aestivum* L. (2n=6x=42). It originated from two distinct interspecific hybridizations among three related diploid species that diverged 5-7 MYA (Marcussen et al., 2014). Interestingly, many other crops are also allopolyploids including cotton, oilseed rape and oat.

3.2.3 Segmantal allopolyploids

In addition to auto- and allopolyploids, Stebbins (1947) proposed a new category of polyploids, known as segmental'allopolyploids. Stebbins used both chromosome behavior and structural divergence concepts in his definition of the term, as at his time chromosome pairing was thought to rely solely on structure, rather than sequence homology (Mason and Wendel, 2020). Segmental allopolyploids are characterized by the formation of both bivalents and multivalents during meiosis, in varying numbers. Therefore, they often resemble meiotic behavior of autopolyploids rather than allopolyploids. Segmental allopolyploids can be defined also as allopolyploids, whose homoeologous chromosomes share the segments of the same sequence.

3.3 Induction of polyploidy

Polyploidization either occurs spontaneously or can be induced artificially. Polyploids are most frequently formed when meiotic irregularities cause the formation of gametes that have more than one set of chromosomes (unreduced gametes). The frequency of unreduced gametes varies from 0,1% to 2% and can be even increased in the response to stress such as drought, low or high temperatures, and physical damage (Svačina et al., 2020). Alternatively, polyploidization can be achieved by somatic doubling.

3.3.1 Natural induction of polyploidy

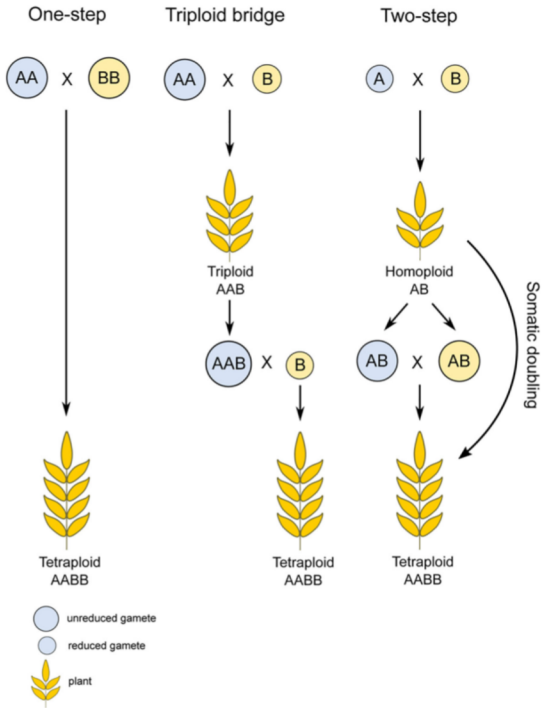

Figure 2: Diagram of natural induction of polyploidy (adopted from Svačina et al., 2020)

There are several ways leading to polyploidy and I will summarize their principles in the following chapters.

3.3.1.1 One step

Polyploids are formed directly in diploid population by the union of two unreduced (diploid) gametes or by somatic doubling. Mitotic nondisjunction supporting spontaneous doubling of chromosomes can occur throughout the life cycle of a plant and may potentially result in mixoploid organisms at the origin of polyploid meristematic cells that ultimately lead to a new polyploid organism (Tayalé and Parisod, 2013). The spontaneous doubling was found for example in cabbage and broccoli. Nevertheless, the somatic doubling is rarely observed under natural conditions and is usually achieved only by chemical treatment.

3.3.1.2 Triploid bridge

Triploid bridge is the most frequent way of polyploid formation, which involves a two-step mechanism of reduced–unreduced gamete fusions (Ramsey and Schemske, 1998). During the first step, a regular haploid (n, reduced) gamete fuses to a rare diploid (2n, unreduced) gamete to form a triploid individual. Then, the primary triploid serves as a "bridge" between diploids and tetraploids. Unreduced gametes of triploids may fuse with reduced gamete of diploid parent and form tetraploid individual (Hojsgaard, 2018). Triploid bridge may lead to the formation of both auto- and allotetraploids. Bridge

crossing is a strategy employed for transferring genes between two species with different ploidy levels through transitional fertile allopolyploids (Sattler et al., 2015).

3.3.1.3 Two steps

Despite being documented experimentally, this pathway is considered far less common than the unreduced gamete pathways (Ramsey and Schemske, 1998). The two-step pathway of allopolyploid creation first involves formation of a homoploid hybrid. Such an individual would require a somatic doubling event, which occurs when mitotic abnormalities result in polyploid somatic cells in a hybrid. If these cells eventually form a generative tissue, self-fertilisation event may result in the development of a polyploid plant. Alternatively, when the progenitors are autopolyploids, an allopolyploid can emerge immediately through the fusion of their standard (i.e., reduced) gametes (Pelé et al., 2018).

3.3.2 Artificial induction of polyploidy

In the plant kingdom, induction of polyploidy has been often used for developing new plant cultivars and is also deemed as a hot topic for research. Therefore, polyploidy is often induced artificially.

3.3.2.1 Induction of polyploidy by radiation

Plants exposed to gamma radiation face noxious effects that are either directly or indirectly induced through oxidative stress. Ionising radiation can directly cause DNA demage by energy deposition leading to excitations and ionisation events that can induce a spectrum of chromosome rearrangements and modifications. Plants require a highly efficient and faithful DNA repair machinery to protect DNA integrity. As such, when a DNA error is detected by DNA checkpoints, cell cycle progression can be delayed or stopped to allow time to repair the damage. (Britt, 1996). Cyclin-dependent kinases (CDKs) are mitotic regulator that drive unidirectional and irreversible progression from one cell cycle phase to the next by phosphorylating target proteins. The genome is replicated during the synthesis phase (S-phase) and is afterwards halved during the final step of the mitotic cell cycle (M-phase) (De Schutter et al., 2007). Transition to S-phase and M-phase can only happen if DNA repair has occurred. Under specific circumstances, M-phase can still proceed without subsequent chromosome separation and cytokinesis ending in formation of polyploid cells (De Veylder et al., 2011). This process, called endoreduplication, is well known in various plant tissues to achieve growth by cell expansion. This mechanism was observed for instance in *Lemna minor* (Van Hoeck et al., 2015).

3.3.2.2 Induction of polyploidy by temperature shocks

At the moment when the dividing cell is exposed to a sudden change in temperature (heat shock, cold shock), mitosis is blocked at metaphase, which results in doubling of its chromosome number (e.g., from diploidy to tetraploidy). If the shock affects a dividing zygote or a very early proembyo, a plant with a doubled chromosome number is produced. Tetraploids were produced for example in maize after high temperature treatment (D'Amato, 1997).

3.3.2.3 Induction of polyploidy by injury

Plant cells have a property of totipotency. When the part of the plant, usually meristematic zone is injured, the cells at that point grow rapidly and form a callus. Coumarin, a chemical compound produced by plants, induces the callus growth. Callus is genetically highly variable tissue, and in some cells, somatic doubling of chromosomes appears. Therefore, vegetative buds developed from the callus are frequently polyploid. Tetraploids were developed in a *Solanum* and *Nicotiana* by this method (Hassan and Rehman, 2017).

3.3.2.4 Induction of polyploidy by nitrous oxide

Nitrous oxide gas (N_2O) can be used to produce polyploid plants, but the mechanism of its action is so far not well understood. After the treatment with N_2O, microtubules are effectively depolymerized. This prevents chromosomes from moving to the poles, resulting in chromosome retention in the center of N_2O-treated cells. Metaphase plate formation took place without delay, however, yielding one daughter cell with a diploid genome, whereas the other (unviable) daughter cell is without chromosomes. This process was seen in *Lilium* spp 'Asiatic hybrid lilies' (Kitamura et al., 2009).

3.3.2.5 Induction of polyploidy by chemical treatment

There are various chemicals that are used for the induction of polyploidy in plants, e. g. colchicine, oryzalin, trifluralin and amiprophos-methyl. Colchicine is an alkaloid extracted from meadow saffron (*Colchicum autumnale* L.) and it is the most widely used antimitotic agent for polyploidy induction (Planchais et al. 2000). The mechanism of action of colchicine involves its binding to α- and β-tubulin dimers, inhibition of microtubule polymerization during the cell cycle and prevention of chromosome/chromatid migration during anaphase. Consequently, cytokinesis is also compromised, resulting in the formation of cells with doubled chromosome number. Colchicine has low affinity to plant tubulins and must be used at millimolar concentrations for effective polyploidy induction in plants (Dhooghe et al. 2011). Artificial plant polyploidy may also be accomplished with other classes of antimitotic agents, such as the herbicides dinitroanilines (trifluralin and oryzalin) and phosphoric amides (amiprophos-methyl and butamiphos), which have higher affinity for plant tubulins. Therefore, micromolar concentrations of such agents might produce the same results as colchicine at millimolar

concentrations (Planchais et al. 2000). This treatment is used for polyploidy induction in many plant species including banana.

3.4 Advantages and disadvantages of polyploidy

There are several advantages of being polyploid including heterosis (in allopolyploids), gene redundancy and switch to asexual reproduction. On the other hand, genomes with multiplied sets of chromosomes face several hurdles before stabilization of genomes including the disrupting effects of nuclear and cell extension, the propensity of polyploid mitosis and meiosis to produce aneuploid cells (presence of abnormal number of chromosomes in a cell) and the epigenetic instability that may result in transgressive gene regulation.

3.4.1 Advantages

3.4.1.1 Heterosis

Heterosis (hybrid vigor) refers to the out-performance of hybrids including allopolyploids over their parental species in phenotype traits, such as plant height and yield. Although, heterosis is evolutionarily defined as that the heterozygotes have higher fitness in a population than the homozygotes, it can also be viewed as superior levels of biomass, stature, growth rate, and/or fertility in the hybrid offspring compared to the parents.

Allopolyploids frequently exhibit superior vigor in a comparison with the mean of its two progenitors. Such hybrid vigor, also referred to as heterosis, generally points to increases in biomass, stature, growth rate, and/or stress tolerance of the hybrid. It has been considered one of the most important aspects for crop improvement (Chen 2010). The hexaploid bread wheat (*Triticum aestivum* L.; AABBDD) is an example of natural allopolyploid species, originated through multiple hybridizations. Cytogenetic and genomic findings suggest that an initial cross between *Triticum urartu* Tumanian ex Gandilyan (AA) and *Aegilops speltoides* Tausch. (BB) resulted in the tetraploid hybrid *Triticum turgidum* L. (AABB), a durum wheat. Posteriorly, one or more hybridization events between *Aegilops tauschii Coss*. (DD) and the allotetraploid *T. turgidum* gave rise to the hexaploid bread wheat (Haider 2013). The allohexaploid *T. aestivum* is the most widely cultivated species of wheat, exhibiting desirable features for bread making, followed by allotetraploid *T. turgidum*, which is the most suitable for pasta production (Pauly et al. 2013) (taken from Sattler et al., 2016).

For viable hybrids, the degree of heterosis is proportional to the genetic differences in two parental strains (East, 1936). In other words, the levels of heterosis increase with increased genetic distances between the parents. For example, hybrids between radish (*Raphanus sativus*) and cabbage (*Brassica oleracea*) exhibit extensive biomass heterosis (Karpechenko, 1927), as do hybrids between a wild tomato species, *Solanum pennelli*, and cultivated tomato, *Solanum lycopersicum* (Eshed and Zamir, 1995).

3.4.1.2 Gene redundancy

The advantage granted by the gene redundancy is the masking of deleterious recessive alleles by dominant wild-type alleles. This can act at two life stages: gametophyte (with halved genome) and sporophyte. Although gametophyte of the organism has reduced complexity, its function still requires the activity of many genes. Once deleterious recessive allele occurs in diploid organism and is masked in its sporophyte by dominant allele, half of the gametes will be non-viable. In contrast, such allele is still masked in gametes of polyploid by wild-type allele(s). In sporophyte, polyploidy can reduce the incidence of recessive homozygotes: whereas diploid Aa heterozygotes produce 1/4 aa homozygotes, AAaa autopolyploids and AaAa allopolyploids produce 1/16 aaaa homozygotes and no aaaa homozygote can be found among the progeny of AAAa plant (Comai, 2005). The protective effect of polyploidy against noxious recessive mutations and genotoxicity can be important when isolated and severely narrowed populations are forced to inbreed, at times when the purification of damaging alleles is difficult by the reduced number of breeding individuals.

The other advantage of gene redundancy is the ability to diversify gene functions by altering redundant copies of duplicated genes. This process is called neo-functionalization. In diploids, such ability is suspended at the appearance of rare segmental duplication events. On the other hand, all genes are duplicated in polyploids and they are available for evolutionary experimentation.

3.4.1.3 Loss of self-incompatibility and gain of asexual reproduction

There are two ways how can polyploidy affect sexuallity and it may provide selective advantages. First, disruption of self-incompatibility systems, and thus allowing self-fertilization has been frequently observed in newly developed polyploids. However, molecular basis of this response is indeterminate. In *Arabidopsis* allopolyploids, it seems that it results from interactions between the parental genomes. Likewise, in autopolyploid of *Petunia hybrida*, it could result from interallelic interactions in the 2x pollen. Second, switch from sexual to clonal reproduction has been evidenced in many polyploid lineages. This can provide selective advantage, as asexual reproduction enables reproduction in the absence of mating partners (Comai, 2005).

3.4.2 Disadvantages

3.4.2.1 Changes in cellular architecture, and regulatory implications

Doubling the genomic content of an organism is usually associated with an increase of its cell volume. This brings consequent change in the relationship between the tridimensional and bidimensional components of the cell. An example of crucial components having different dimensional properties is granted by chromatin and the nuclear envelope. According to the relationship between volume and surface of a sphere, doubling the genome is expected to double the volume that is occupied by chromatin, but causes only a 1.6-fold increase in the nuclear envelope surface.

This difference, although apparently modest, can change the stoichiometry of the interaction between components of chromatin that are located at the nuclear periphery and envelope-bound proteins, because tridimensional organization of chromosomes in the nucleus involves the peripheral positioning of telomeric and centromeric heterochromatin (Fransz et al., 2002).

On the other hand, increased amount of DNA and related increased cell volume can be advantageous for cells that have high metabolic rates. Besides the potential for regular polyploidy, most organisms, from bacteria (Akerlund et al., 1995) to eukaryotes (Sugimoto-Shirasu and Roberts, 2003), can also modulate the amount of DNA in their nucleus by experiencing DNA endoreduplication, which leads to larger, endopolyploid cells.

3.4.2.2 Difficulties in mitosis

Polyploidy may cause problems for the normal completion of mitosis and meiosis. Autotetraploid yeast shows an increased mitotic loss of chromosomes, which results in aneuploid cells (Mayer and Aguilera, 1990). The spindle irregularities can cause difficulties in mitosis. There is lack of information about the mitotic stability of polyploid plant cells. Organization of the plant mitotic spindle does not depend on centrosomes and although knowledge on the dynamics of mitotic spindle formation is emerging (Chan et. al, 2005), the response of such system to polyploidy is so far largely unknown. Although the susceptibility of autopolyploids to the mitotic production of aneuploids might vary from taxon to taxon, the available data indicate the existence of a considerable risk of aneuploidy.

3.4.2.3 Difficulties in meiosis

3.4.2.3.1 Autopolyploids

Autopolyploids possess three or more almost identical sets of chromosomes (homologues). This opens a way for the formation of multivalents at the first meiotic division. The segregation of chromosomes bound in trivalents, quadrivalents and other multivalents may significantly differ from those paired in bivalents. The quadrivalent can produce abnormal segregation patterns, such as '3:1 'or '2:1 plus one lagging chromosome'. This may lead to the development of aneuploid gametes, which are frequently sterile or display reduced fitness. If aneuploid gamete participates to the next generation, aneuploid progeny may significantly increase the genome instability of the population. For this reason, it is believed that chromosome pairing in strictly bivalent manner is an adaptation that stabilizes polyploids (Santos et al., 2003). Another hypothesis indicates that a transition to bivalent pairing is not necessary and the 2:2 segregation of quadrivalents can be achieved by unknown mechanisms that favour this type of segregation. Nevertheless, mechanisms that are required for normalization of autotetraploid meiosis have an important role in adaptation because newly established autotetraploids frequently produce aneuploids.

Irregular meiosis can be seen in triploids, pentaploids and other odd number polyploids. They are formed from union of gametes of different ploidy, such as 1x and 2x, frequently by the merge of reduced and unreduced gametes. In triploids, chromosomes involved in trivalents cannot be segregated into balanced products, and random segregation produces mostly aneuploid gametes. Rely on the species, aneuploid gametes (or gametophytes) and the resulting zygotes vary in viability.

3.4.2.3.2 Allopolyploids

Allopolyploids originate from the merge of gametes from two distinct species. Thus, they possess three or more sets of chromosomes, which more or less differ (homoeologues). For the stable meiosis and the production of gametes with balanced composition of parental genomes, formation of bivalents from homologues is required, because intergenomic recombination compromises the maintenance of the two parental chromosomal complements. The strict pairing of homologous chromosomes is enforced either by sequence dissimilarities of the homoeologous chromosomes or by the existence of molecular mechanism hampering pairing of homoeologues. In allohexaploid wheat, a locus called *Ph1 (Pairing homoeologous 1)* is required to avoid homoeologous pairing and is believed to be a result of an adaptation to polyploidy (Prieto et al., 2004). Other systems exist in various allopolyploids including *PrBn* gene in *Brassica napus* (Jenczewski et al., 2003).

As was mentioned above, aneuploid gametes can be produced during polyploid meiosis. However, the frequency varies between species and depending on polyploidy type. This means that there is a casual relationship between polyploidy and aneuploidy. In fact, eupolyploids may produce frequent aneuploids, which in turn can produce euploids (Henry et al., 2005). The possibility of the occurrence of aneuploidy is relevant because it can trigger epigenetic and genomic instabilities (Matzke et al., 2003). Aneuploidy can cause epigenetic changes because of the sensitivity of chromatin regulatory pathways to the dosage of genes that encode regulatory factors (Schotta et al., 2003).

3.4.2.4 Epigenetic instability

Epigenetic instabilities are frequently associated with the process of diploidization. This phenomenon refers to the process of reverting polyploid state back to diploid. It has been assumed that genetic diploidization involves slow processes of gene inactivation mediated by mutation or progressive methylation. Genomic structural changes, providing diploid meiotic behavior, can be instant and involve sequence elimination or rearrangement soon after polyploid formation. It was suggested that changes in gene expression resulting from epigenetic gene silencing can also occur directly following the creation of a polyploid.

In Arabidopsis, a novel type of epigenetic silencing that occurs solely as a consequence of a change in ploidy level has been discovered. A single copy transgene that was expressed in a diploid plant became silenced in 6–18% of triploid progeny that were produced by crossing the diploid

with a tetraploid wild type. The reversibility of this gene inactivation demonstrated that epigenetic processes were involved (Matzke et al., 1999). A similar type of epigenetic silencing involving random methylation changes of low copy coding sequences was identified in newly synthesized allopolyploid wheat (Mittelsten Scheid et al., 1996).

3.4.2.4.1 Autopolyploids

The instability is likely connected to genome doubling. There is an example when epigenetic effects on transgenic locus were compared between diploid and tetraploid *A. thaliana* (Mittelsten Scheid et al., 2003). The transgene (R) was subject to silencing and the silenced epigenetic state (r) was stably inherited in both diploids and tetraploids. On the other hand, a diploid heterozygote (Rr) produced the equal ratios of silenced alleles (r) and active alleles (R), whereas a tetraploid heterozygote (RRrr) produced gametes preferentially with silenced alleles (rr). Thus, the Mendelian rule of allelic segregation has been violated (Mittelseten Scheid et al, 1996) (taken from Comai 2005). This behaviour of observed locus indicates the effect of ploidy on chromatin remodeling. It is unclear how widespread phenomenon it is and what feature of autopolyploidy is responsible for this observation. The chosen locus does not display any characteristics of epigenetic instability. Besides above-mentioned transgene in Arabidopsis, other loci violating random segregation in polyploids including the gene for activation of a DNA transposon of the Spm/CACTA family have been identified (Wang et al., 2004; Madlung et al., 2004).

3.4.2.4.2 Allopolyploids

Further evidence for rapid epigenetic gene silencing in polyploid genomes comes from an investigation of nucleolar dominance in plants. This phenomenon, observed in interspecific hybrids or allopolyploids, concerns the expression of rRNA genes exclusively from one parent (Pikaard and Chen, 1998). These epigenetic changes of rDNA expression are developmentally regulated and correlate with DNA methylation and histone deacetylation. Nucleolar dominance appears to reflect a specific response to allopolyploid formation because the same bias toward one set of parental rRNA genes is also seen in natural allopolyploids derived from the same diploid progenitors.

3.4.2.5 Cytonuclear interaction

Genetic information in a plant cell is divided between the nuclear and cytoplasmatic genomes. This disposition of multiple genomic compartments did persist through billions of years of evolution. The functional interactions between nuclear and cytoplasmic genomes have major evolutionary repercussion. Mitochondria and plastids have been transitioned from free living organisms to organelles. After that, the dominant subject in eukaryotic genome evolution is the extreme reduction of cytoplasmic genomes. There is only small number of key genes which have been preserved in mitochondrial

and plastid genomes. Furthermore, number of mitochondrial and plastid genes encodes components involved in protein complexes consisting of products of both organellar and nuclear genes.

It is evident that polyploidization of genome disrupts stoichiometry between nuclear and organellar components of these protein complexes because doubling of nuclear genes is not followed by the duplication of the organellar genes. The nuclear genome is stored in nucleus in form of linear molecules called chromosomes, unlike the mitochondria and chloroplast genetic information, that is stored in organelles. There is only one nucleus in each cell, but the number of organelles can vary greatly (Sharbrough J et al. 2017). The genetic information of plastids (called plastome) can be circular or linear, mono- or multimeric (Lilly JW et al. 2001). In most plant taxa, the plastome is formed by circular molecule between 108 and 218 kb in length and is highly conserved among terrestrial plant species. Plastomes are generally inherited maternally (in about 80% of angiosperms), however there were observed causes of biparental or paternal inheritance (Birky CW, 1995). Number of chloroplasts per cell is known to vary between cells of the same species, organism, or even tissue, similarly to the number of cpDNA molecules per each chloroplast.

In contrast to the plastome, the mitochondrial genome is highly variable in higher plants. It ranges from 208 kbp in white mustard (Brassica hitra) to over 11,000 kbp in *Silene conica*. Number of genes ranges between 50 and 73, including 30 to 37 protein coding genes (Burton RS et al., 2013; Sloan DB, 2013). Suchlike to plastome, the plant species in majority inherit the mitochondial genome maternally. There are still some exceptions when it is inherited paternally or biparentally. Similarly to plastome, there is large variability in the number of mitochondria per cell and the number of mtDNA molecules per mitochondria (Sharbrough J et al., 2017). Interestingly, it seems that the number of mitochondria frequently exceeds the number of mtDNA copies per cell (Preuten et al., 2010).

The intimate communication between nucleus and organelles are through the anterograde (from nucleus to organelles) and retrograde (from organelles to nucleus) signaling. Moreover all known transcription factors regulating organelle transcription are encoded in the nucleus (Woodson et al., 2008). Thus, it is evident that the regulation of gene expression is directly controlled by the nucleus. Above that, core eukaryotic functions hinge on integration and coevolution between nuclear and organellar genes. The level of integration extends down to direct molecular interactions within multisubunit enzyme complexes (Woodson et al., 2008, Rand et al., 2004). For instance, essential enzymes in mitochondria and plastids, such as oxidative phosphorylation complexes, the photosynthetic apparatus, and organellar ribosomes, are composed of gene products from both nuclear and organellar genomes (Forsythe et al., 2019). Such example is Rubisco, a protein complex whose large subunit is encoded by nuclear gene *rbcL*, while small subunit is encoded by chloroplast gene *rbcS* (I. Andersson, A. Backlund, 2008). There are also interactions between cytoplasmic RNAs and nuclear-encoded proteins that are responsible for post-transcriptional processes, including intron splicing, transcript end processing, and RNA modification (Germain et al., 2013). From this, it is obvious that the cytonuclear interactions are crucial for proper functions of plant genomes and each stoichiometry disturbance

between the nuclear-encoded and the organelle-encoded genes can lead to the genome malfunction and reproductive isolation in many systems (Sloan et al., 2017).

The balanced relationship between the nuclear- and organelle-encoded genes can be disturbed by whole genome changes, including polyploidization. Given a whole genome duplication (WGD) duplicate the number of nuclear-encoded genes, the number of organelles is not proportionally increased. This opens a question whether nuclear genome duplication modify the stoichiometry between nuclear-encoded and organelle-encoded subunits of protein complexes, or if intergenomic coordination is able to compensate for doubling copy number of nuclear genes involved in cytonuclear complexes, such as Rubisco. This compensation may be in form of increased number of organelles, increased copy numbers of cpDNA/mtDNA in each organelle, increased expression of organelle-encoded genes and/or decreased expression of nuclear-encoded genes involved in cytonuclear complexes. So far, there is only scarce information, how polyploids response to the disturbed stoichiometry. In autotetraploid *Arabidopsis thaliana*, the copy number of chloroplast genes decreased slightly per nuclear genome (0.76-fold) compared to diploids, whereas the copy number of mitochondrial genes doubled. However, it is not revealed if this doubling is due to an increase in the number of mitochondria per cell or because of the number of mtDNA per mitochondria (Coate et al., 2020). Using the RNAseq approach, it was found that total nuclear transcription targeted to plastids decreased with increased ploidy level, while that targeted to mitochondria increased with raising ploidy. This indicates that the coordination of transcription between the genes, which are nuclear-encoded and these which are organelle-encoded is more conserved across ploidy levels for plastids and less conserved for mitochondria (Coate et al., 2020). In the long term, organelle-targeted nuclear genes appear to be the first and most common group of nuclear genes returning to a single copy after polyploidization.

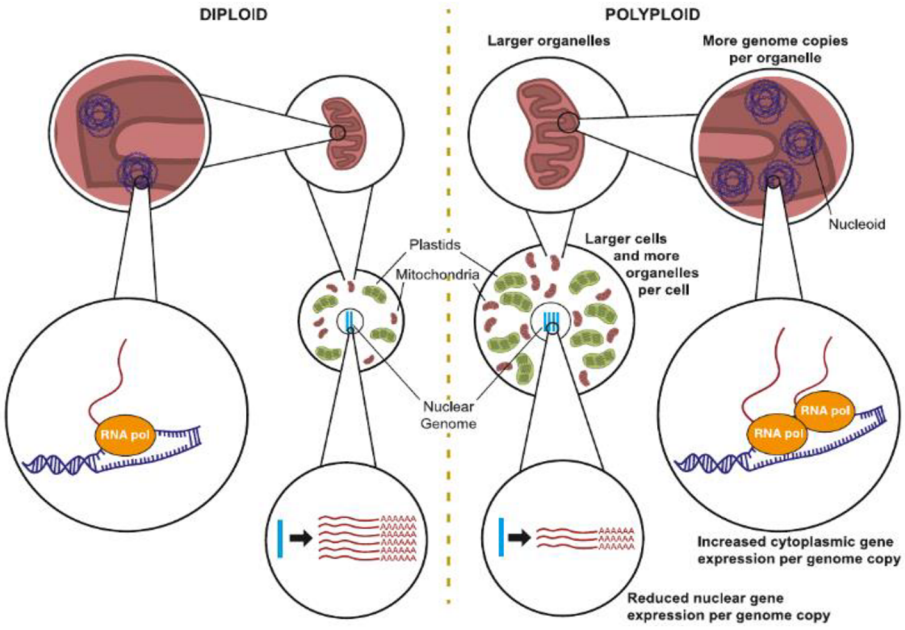

Figure 3: Disruption in stoichiometry between nuclear- and organelle-encoded genes involved in chimeric complexes after polyploidization could be solved by various mechanisms (Adopted from Sharbrough et al. 2017)

Another challenge for cytonuclear interaction can be seen in interspecific hybrids and allopolyploids. For the cytonuclear genome, there is typically uniparental inheritance (usually maternal), however the nuclear encoded genes come from two distinct parental genomes (Sharbrough et al., 2017). If the parental alleles of these organelle-targeted nuclear genes or their promoter domains are sufficiently divergent, one would expect preferential expression from the maternal allele, as well as higher retention of maternal allele over evolutionary time (Gong et al., 2014, Sehrish et al., 2015). Indeed, the maternal RuBisCo has been preferably expressed in wild and cultivated cotton, as in resynthesized F1 hybrids, compare to the paternal homoeologue (Gong et al. 2012). On the other hand, this skewed expression of the maternal homoeologue was not found in the allopolyploid *Tragopogon miscellus* and the resynthesized allopolyploid *Oryza sativa* (Sehrish et al., 2015, Wang et al. 2017). From the above mentioned studies, it is evident that many aspects of the polyploid formation and evolution including the responce to disturbed stoichiometry in cytonuclear interaction has to be revealed and understood. My thesis contributes to sheding the light on these phenomena.

3.5 Studied plants

3.5.1 Italian ryegrass (*Lolium multiflorum* Lam.)

Italian ryegrass is outcrossing, strongly competitive and fast-growing species of forage grass, capable of producing large quantities of seeds. It is genetically diverse and shows a high degree of phenotypic plasticity, which means that it is a highly adaptive plant.

It is sensitive to frosts, does not tolerate high groundwater levels, summer drought and usually suffers from snow mold under snow cover.

The natural distribution of this species covers Central and Southern Europe, Northwest Africa and Southwest Asia (Hubbard, 1968), however it is now distributed in temperate regions of all continents, mainly due to pasture cultivation. The species is valued as an important forage component. While usually restricted to lowland areas, it can grow even at higher altitudes up to 900 m above sea level under suitable conditions (Beddows, 1973).

3.5.2 Meadow fescue (*Festuca pratensis* Huds.)

Meadow fescue is a typical representative of mesophilic meadows and pastures. It is one of the most valuable forage species and is a component of grass mixtures for temporary to perennial meadows and pastures, where it ensures production in the first three years. It has relatively good yield and forage quality.

This species has a very good root system and tolerates biotic and abiotic stresses, including winter freezing. The plant is especially suitable for mowed pastures, wetlands and peat soils and performs well in North European conditions and highland and mountain areas of Central Europe. The disadvantage

of this plant is its low persistence and low competitiveness against weeds due to the slower initial development.

3.5.3 *Festulolium*

Festulolium is a hybrid originated from the mating of representative of ryegrass (*Lolium*) with the representative of fescues (*Festuca*). This hybrid can be found in nature, such as along riverbanks in Wales and England (Humphreys and Harper). However, these natural hybrids are sterile. Complementary characteristics of both parents, rapid establishment, high yield and nutrition of ryegrasses and tolerance to abiotic stresses and persistence of fescues, initiates the breeding'efforts to combine these features in a single organism. In the seventies of the last century, breeders succeeded in releasing the first Festulolium cultivars and since then, many breeding stations developed almost 100 cultivars registered on the OECD (Organisation for Economic Co-operation and Development) list. They became popular among farmers and are frequently used as a component of mixtures used for grazing, hey and silage production as well as in turf mixtures. It is worth mentioning that the most successful breeding program is located at DLF Seeds, a breeding station in Hladke Zivotice, Czech Republic.

4 Materials and metods

4.1 Materials and tools

In following chapter, I will describe materials and tools used in this project.

4.1.1 Laboratory equipment

For development of this work, were used these tools:

- automatic pipet (Nichiryo)
- biological thermostat BT 120 (Labo MS spol. s. r. o.)
- centrifuge myFuge Mini (Benchmark Scientific)
- cryostat (Leica)
- confocal microscop (Leica)
- droplet generator (Bio-Rad)
- droplet reader (Bio-Rad)
- electrophoretic chamber Wide Mini-Sub Cell GT Cell (Bio-Tech)
- laboratory weighing-machine (OHAUS)
- magnetic stirrer Variomax (Electronicrührer)
- microwave oven (Zanussi)
- Nanodrop ND-1000 Spectrophotometer (Thermo Scientific)
- phytotron chamber (Wiss Gallenkamp)
- Qubid 3 fluorometer (Thermo Fisher)
- refrigerated centrifuge (VWR International)
- thermocycler C100 Touch (Bio-Rad)
- transilluminator InGenius LHR (Syngene)
- voltage source PowerPacTM (Bio-Rad)
- water bath SUB6 (Grant)

4.1.2 Used software

For evaluation and processing of data were used following computer programs

- LaxX
- Imaris
- Microsoft Excel
- Geneious

4.1.3 Chemicals

The following chemicals were used t. The composition of the buffers is summarized in Table 1.

- 1x TAE buffer
- 6X DNA Loading Dye (Thermo Fisher Scientific, cat. n. R0611)
- 10X DreamTaq Green Buffer (Thermo Scientific, cat. n. EP0714)
- agarose (Amresco, cat. n. 0491B70)
- antifade mounting medium with DAPI (Vectashield, cat. n. H-1200-10)
- Cryo-Gel (Leica, cat. n. 39475237)
- ddPCR droplet reader oil (BioRad, cat. n. 1863005)
- ddPCR Supermix for Probes (BioRad, cat. n. 1863024)
- ethidium bromide (Top-Bio, cat. n. P047)
- GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Fisher Scientific, cat. n. SM1333)
- immersion oil (Leica, cat. n. 11944399)
- nuclease-free water (Thermo Fisher Scientific, cat. n. 10977015)
- sucrose (Lach-ner, cat. n. 10135-AP0)
- paraformaldehyde (Sigma-Aldrich, cat. n. 30525-89-4)

4.1.3.1 Chemical kits

- nucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, cat. n. 740609.250)

Table 1: Composition of used buffers

Buffer	Ingredient	Concentration
1x TAE pH 8	Tris	0,04 $mol \cdot l^{-1}$
	EDTA	0,02 $mol \cdot l^{-1}$
	H_2O	0,001 $mol \cdot l^{-1}$

4.1.3.2 Enzymes

During the work on my Bc. theses, I used these enzymes:

- DreamTaq Green DNA Polymerase (Thermo Scientific, cat. n. EP071)
- HindIII (New England BioLabs, cat. n. R0104S)
- BsmI (New England BioLabs, cat. n. R0134S)

4.1.3.3 Oligonucleotides

Oligonucleotides used for PCR amplification are summarized in Table 2 and oligonucleotides used for ddPCR amplification are in Table 3. All oligonucleotides were design by Joanna Majka Ph.D.

Table 2: Oligonucleotides used for PCR

Name	Sequence (5' ⟶ 3')
*atpI*_F	CTTGGATTATTTACAAGCG
*atpI*_R	CTCCATGGATTCACCTATA
*atpA*_F	TGGGTCGTGTTATAAATG
*atpA*_R	AGCAGGAGATTCAATTAAG
*psbD*_F	AACCCAACTCAAGCTGAA
*psbD*_R	GCAACACCAAAGATTTGG
*rbcL*_F	CAACCAATGGATCTGTTA
*rbcL*_R	CCCACAATAGAAGTAAACA
*ndhC*_F	TTGGCATTTTGGATTTCA
*ndhC*_R	ACCCGATTCATAACTAGAA
*atpC1*_F	CTCGTCTACTCCAAGTTC
*atpC1*_R	TCTCGATCTTGATCTTCTC
*psbQ1*_F	CATCGACAGGAAGCAATG
*psbQ1*_R	GAGGCTCTTCTTCTCCTC
*rbcS1*_F	GCATCAAGAAGTTCGAGA
*rbcS1*_R	CTTCCACATTGTCCAGTA
*rbcS2*_R	CACGGAAGATGAAACCAA
*ndhL*_F	TCACCTACCTCTTCTTCC
*ndhL*_R	GGTACTTCATGGTTGGATC
*cox2*_F	GTTCCACGAATCTCACTG
*cox2*_R	GTACCTGGTCGTTCAAATC
*nad6*_F	CCGGTACATTCCGTTTTG
*nad6*_R	TCGAGACCTAACAAAATAAGTA
*cox15*_F	CACTGAATCCAACAGTGG
*cox15*_R	GACTCCACCAAGTATGAC
*nad7*_F	CTCGATGGGTTATCTCTA
*nad7*_R	GGAGAACACCATACAGTA
*matK*_F	CCCTATCCTATCCATTTG
*matK*_R	ATCGCAATAAATGCAAAG

*matR*_F	TCCCCATACAGATAGAGG
*matR*_R	GGTCTTCTTCGGCTAATG

Table 3: Oligonucleotides used for ddPCR

Name	Type of signal	Sequence (5' ⟶ 3')
atpI	FAM	TGCAACATTAGCCGCAGCCT
	HEX	TTGCAACGTTAGCCGCAGCC
atpA	FAM	CTTTCCCATCAATAGGTTTAGCCAAA
	HEX	ACAATTTCGCCTCTCCCATCAATAG
psbD	FAM	ACTTATTCAATGGTCACGGCTAACC
	HEX	TCAATGGTCACTGCTAACCGC
rbcL	FAM	CCTATTTGAAGAAGGTTCCGTTACT
	HEX	AGTAACGGAACCCTCTTCAAATAGG
ndhC	FAM	CTTCTCAGGTCCTTCACTAACCG
	HEX	AGCTTCTCCGGTCCTTCACT
atpC1	FAM	ACGGTGAGCTTGCCTTCCTT
	HEX	ACAGTGAGCTTGCCTTCCTTG
psbQ1	FAM	ATGAACGACCTCCGCCTCAG
	HEX	CATGAATGACCTCCGCCTCAG
rbcS1	FAM	TACCTTATCTGCCACCGCTCT
*rbcS1*_Fp	HEX	CTTACCTGCCATCGCTCTCG
*rbcS2*_Lm	HEX	CTTATCTGCCACCGCTCTCG
ndhL	FAM	TTACAAACGGTGCCCAGAGC
	HEX	AGACTGATGCTCTGGGCACC
cox2	FAM	ACCTCCATCTCGGTACAACGA
	HEX	CTGACCATAGTAAACTCCTTCTCGG
nad6	FAM	TTCCCATCCTAGTCTTTTGCGACA
	HEX	TCCTAGTATTTTGCGACACTTCTGGT
cox15	FAM	AGCACTAAAGCAGCTCGTTACCA

	HEX	AGCACTAAAGCTGCTCGTTACCA
nad7	FAM	CCAATGGTGGCGGCTACTAC
	HEX	CCAATGGTGGTGGCTACTAC
matK	FAM	TTCAACTCCTTCAATACCGTATCCAA
	HEX	TCAACTCCTTCAATACCGTCTCCA
matR	FAM	CCCGAAGCCTTCGGAGTATCTT
	HEX	ATCCCGAAGCCTTTGGAATATCTTT

4.1.4 Plant material

Plants used in this project were grown in a phytotron with conditions were to day and night cycle 16h day and 8h night. During the day the temperature was set at 20 ° C and at night at 16 ° C. Humidity was set at 60%. The list of plants is summarized in the tables below (Table 4-7).

Table 4: Autopolyploids of *Festuca pratensis*

Ploidy	**Cultivar**	**Ploidy**	**Cultivar**
2x	Fure	4x	S2 Moestr
	Tomosake		Raskilla
	Cosmonaut		Patra
	Kolumbus		Tetrax
	Preval		Westa

Table 5: Autopolyploids of *Lolium multiflorum*

Ploidy	**Cultivar**	**Ploidy**	**Cultivar**
2x	Partax	4x	Pepper
	Tiger		Dallara
	Barherta		Passat
	Yolande		Firkin
	Skippy		Mitos

Table 6: Newly synthetized *Festuca* autopolyploids

Ploidy		Ploidy	
2x	Hyperbola (plant no. 6)	4x	Hyperbola (plant no. 5)
	Praniza (plant no. 9)		Praniza (plant no. 8)
	48616 (plant no. 10)		48616 (plant no. 11)
	24189 (plant no. 14)		24189 (plant no. 12)
	Kolumbus (plant no. 17)		Kolumbus (plant no. 19)

Table 7: Homoploids, allopolyploids and their parents (parents of allopolyploids Westa and Mitos are included in Tables 4 and 5)

Ploidy		Ploidy	FpLm	Ploidy	LmFp
2x	Plant no. 3/3	4x	Plant no. 10/2	4x	Plant no. 5/1
	Plant no. 5/3		Plant no. 10/4		Plant no. 5/2
	Plant no. 6/7		Plant no. 10/5		
	Plant no. 7/5				
	Plant no. 8/2				
	Lm MATIZ 74/11				
	Fp WSC 62/11				

4.2 Experimental and evaluation procedures

4.2.1 Evaluation of changes in plant cell before and after polyploidization

4.2.1.1 Preparation of microscopic slides

4.2.1.1.1 Plant tissue fixation and dehydration

From each plant I trimmed one young leave. Leaves were excised and then submerged in a solution containing 4% paraformaldehyde and 1x phosphate-buffered saline (PBS; pH 7.0). Samples were fixed for 2h at room temperature. Then the leaves were subjected to infiltration in sucrose gradient. Gradient infiltration steps with 25%, 33%, 50%, 66%, and 75% 2.3 M sucrose were performed for 1h each at room temperature, whereas the final 100% 2.3 M sucrose was infiltrated overnight at 4°C.

4.2.1.1.2 Cryosectioning

Leaf tissue was embedded into Cryo-gel and frozen at -25°C. Frozen blocks with the samples were trimmed, thick sections were taken and sectioned until the region of interest was reached. The optimal thickness of the sample to be transferred to the microscope slide was determined to be 20 μm. Samples were labelled with fluorescent dye DAPI. The slides were stored in dark at 4°C until imaginig.

4.2.1.2 Evaluation of imaged samples in software Imaris

Images were vizualized in Imaris software (Figure 4). The number of chloroplasts, size and volume of cells, size and volume of chloroplasts and volume of nucleus were examined. Generally, five plants were used for each cultivar and 12 cells from each plant were examined for numbers of chloroplasts, cell size and cell volume. In total, 30 chloroplasts and nuclei from each cultivar were examined for chloroplast size and volume and volume of nucleus. Autopolyploid set contained 20 cultivars containing 100 plants, 50 diploids and 50 polyploids, plus 5 diploids and 5 tetraploids of *Festuca pratensis* plants. These were clones of the single plant treated by colchicine, where one plant was diploid and one plant was tetraploid (five such pairs). Allopolyploid set contained 14 plants, 10 hybrids including five diploid diploid hybrids with their parents and five tetraploid hybrids with their autotetraploid parents.

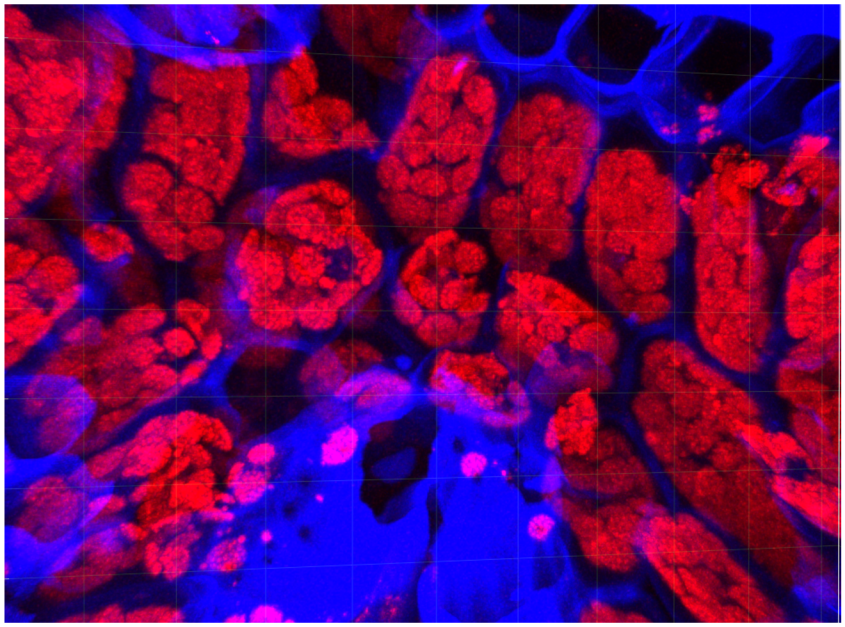

Figure 4: Cells observed in software Imaris

Statistics were generated from the obtained data. The percentage difference between the ploidy of individual species and hybrids was evaluated, and subsequently these percentage differences were averaged. Violin plots were made from statistical data in the Prism GraphPad program.

4.2.2 Determination of gene copy number in auto- and allopolyploids

4.2.2.1 Gene selection

Firstly, we have selected genes of interest (three pairs for chloroplast-nucleus interactions and two pairs for mitochondria-nucleus interactions and three non-intercting nuclear genes as controls). Oligonucleotide primers and probes were designed byJoanna Majka PhD (Table 8).

Table 8: List of selected genes

Chloroplasts	Nucleus	Complex	Mitochondria	Nucleus	Complex	Non-interacting genes
atpI *atpA*	*atpC1*	ATP synthase	*cox2*	*cox15*	Cytochrome c oxidase (complex IV)	matK (chloroplast)
psbD	*psbQ1*	Photosystem II	*nad6*	*nad7*	NADH-ubiquinone oxidoreductase (respiratory complex I)	matR (mitochondria)
rbcL	*rbcS*	Rubisco				actin (nucleus)

4.2.2.2 DNA isolation

Genomic DNA was isolated from all plants. The youngest or second youngest leaves were cut into small pieces and placed in a 2 ml microtubes. Glass beads were added to the microtubes to aid homogenization. The leaves were first freeze dried overnight then crushed using a homogenizer.

The commercial NucleoSpin Plant II kit was used to isolate the genomic DNA. In this way, 100 µl of DNA was isolated and the concentration of this DNA was measured with a nanodrop and diluted to the resulting concentration of about 10 $ng \cdot \mu l^{-1}$.

4.2.2.3 Condition optimization

Appropriate primer annealing temperatures were determined by gradient PCR (Table 9). Temperature steps were 55-65 °C, based on the temperatures recommended by the manufacturer (Table 10).

Table 9: Gradient PCR reaction mix

Stock solution	Stock solution concentration	Working concentration	Volume [µl]
PCR buffer	10x	10x	5
dNTPs	100µM	10µM	2
DreamTaq Green DNA Polymerase	5 $U \cdot \mu L^{-1}$	1 $U \cdot \mu l^{-1}$	0.2
Forward primer	50µM	10µM	1.25

Reverse primer	50µM	10µM	1.25
gDNA	sample dependent	10 ng·µl^{-1}	1
H2O	-	-	9.3
total volume			**20**

Table 10: PCR cycling conditions

Steps	Cycles	Temperatures	Time
Initial denaturation	1	94°C	3 minutes
Denaturation	35	94°C	30 seconds
Aneling		55-65°C	45 seconds
Extension		72°C	1 minute
Final extension	1	72°C	2 minutes

Results of the PCR amplification were visualised by gel electrophoresis in a 1% agarose gel in 0.5 × TBE buffer. 3 µl of 6x Loading Dye and 5 µl of the PCR reaction were mixed in each well.

Separation by gel electrophoresis was performed for 60 minutes at 80 W. Upon completion, the gel was stained in a solution of ethidium bromide for 15 minutes and then washed in deionized water. After staining, the gel was evaluated in the GeneSnap program.

4.2.2.4 ddPCR condition optimization

The genomic DNA was digested with restriction enzymes. Optimal annealing temperatures for oligonucleotide probes and suitable working concentrations of DNA and the probes were determined using the concentration gradient ddPCR.

Table 11: Genomic DNA restriction digest

Stock solution	Stock solution concentration	Used concentration	Volume [µl]
Buffer			5
Enzyme		20 U·µl^{-1}	1
gDNA	sample dependent	sample dependent	5
H_2O	-	-	39
total volume			50

According to the number of analyzed samples, the amount of reaction mixture was prepared for ddPCR according to the schedule (Table 12).

Table 12: ddPCR reaction mix

Stock solution	Stock solution concentration	Work concentration	Volume [µl]
Super mix for probes	2x	1x	11
Forward primer	50µM	10µM	1
Reverse primer	50µM	10µM	1
Hex probe	250 ng·µl^{-1}	125 ng·µl^{-1}	0.257
Fam probe	250 ng·µl^{-1}	125 ng·µl^{-1}	0.257
gDNA	sample dependent	0,1/ 5 ng·µl^{-1}	1
H_2O	-	-	7.45
total volume			**22**

Droplets were generated as follows: A droplet generating cartridge was inserted and snapped into the stand (DG8TM QX100TM / QX200TM Drop Generator Cartridges). 60 µl of probe oil was pipetted into the lower wells (labeled "Oil"). 20 µl of the reaction mixture and DNA were gently pipetted into the middle wells (pipetted at an acute angle so as not to form bubbles in the well). After checking and removing the bubbles, the rubber band was then pulled over and the cassette was inserted into the droplet generator. After droplet formation, 40 µl of the emulsion mixture (reaction mixture and oil) was aspirated from the upper wells of the cartridge very gently at an obtuse angle and then slowly discharged to the wall of the bottom of the well of the ddPCR 96-well plate. The process was repeated until all samples analyzed were placed in the ddPCR plate. The plate was closed using pierceable aluminum sealing foil, placed in a deepwell thermocycler and the program for ddPCR was started (Table 13).

Table 13: ddPCR cycling conditions

Steps	Cycles	Temperatures	Time
Initial denaturation	1	95°C	10 minutes
Denaturation	35	94°C	30 seconds
Aneling		59-65°C	1 seconds
Extension		98°C	10 minute

The ramp speed 2°C/sec

After completion of the PCR program, the plate was inserted into the analyser holder, and after setting up the analytical program according to the number of samples, the analysis was started. Following the completion of the program, the number of droplets positive for FAM (signal for *Lolium*), and HEX (signal for *Festuca*) were compared, and the gene copy number in the plant was analytically calculated for both signals and their ratios. If the analyzer was not able to automatically evaluate the result, a positive response threshold was set manually.

Droplet Digital PCR can be viewed as a 1-D plot, where each droplet from the sample is plotted on the graph of fluorescence intensity vs. droplet number. In Figure 5, are droplets above the pink threshold line scored as positive, each assigned a value of 1. All droplets below the pink threshold line are scored as negative, each assigned a value of 0.

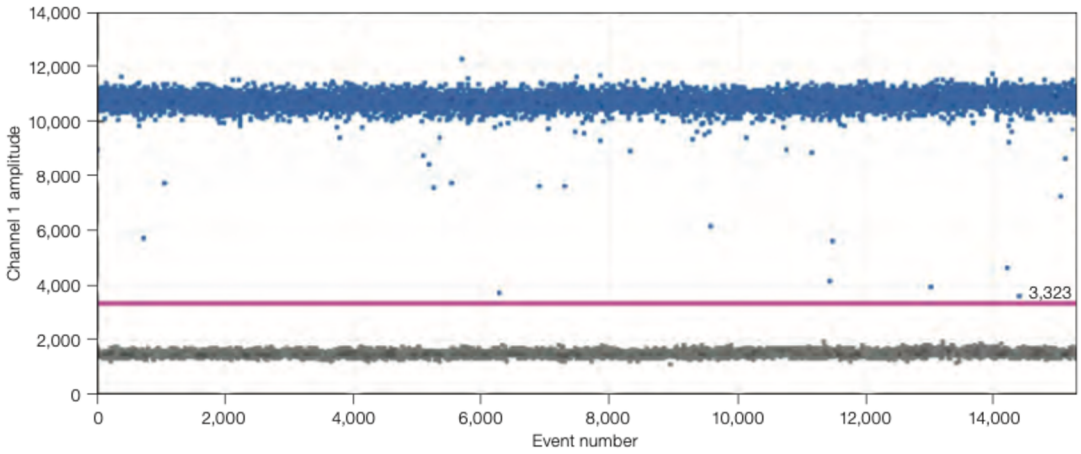

Figure 5: Each droplet from a sample is plotted on the graph of fluorescence intensity vs. droplet number

5 Results

5.1 Evaluation of changes in the plant cell before and after polyploidization

In the submitted bachelor thesis the intracellular changes associated with polyploidization were evaluated. Overall, I evaluated 122 plants from which 110 were autopolyploids and 10 were allopolyploid plants complemented with their parents. Results were visualized using violins plots. The dashed line represents the median and dotted lines represent lower and upper quartiles. The width of the graph indicates the number of individuals of a given volume or number (Figure 5-8).

Firstly, I evaluated set autopolyploid of plants (Tables 4 and 5) and newly established autopolyploids (Table 6). Generally, 60 cells per cultivar were examined for chloroplast number and cell volume, 30 were examined to evaluate the volume of nucleus and volume of chloroplasts.

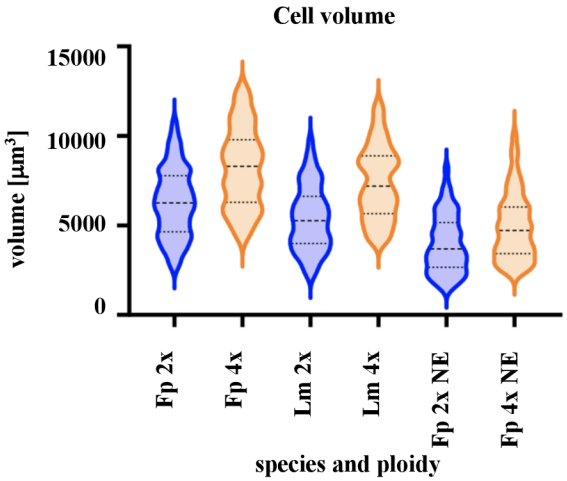

Figure 6: Visualization of the distribution of the obtained data for cell volume. Fp NE referes for newly synthetized *Festuca pratensis* autopolyploids

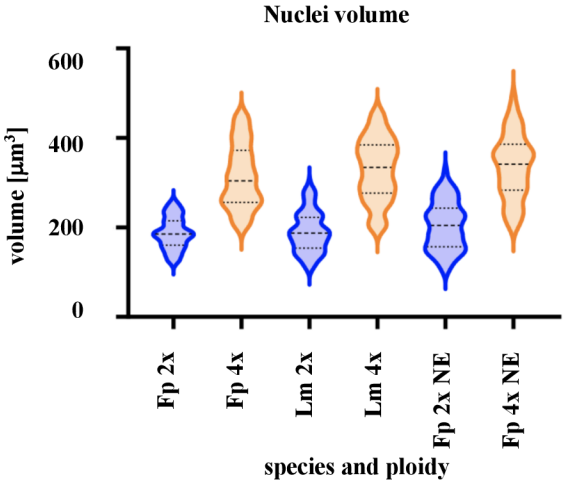

Figure 7: Visualization of the distribution of the obtained data for nucleus volume

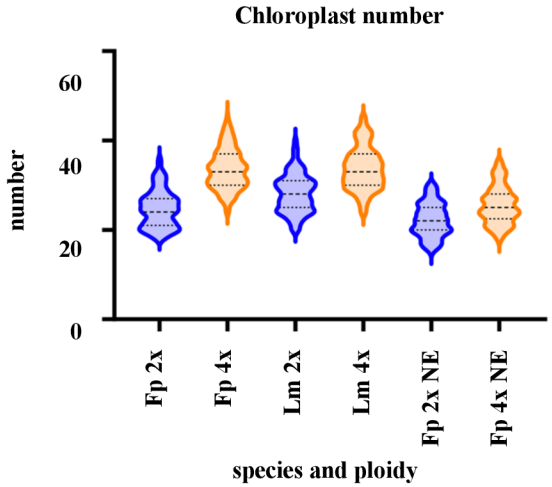

Figure 8: Visualization of the distribution of the obtained data for chloroplast number

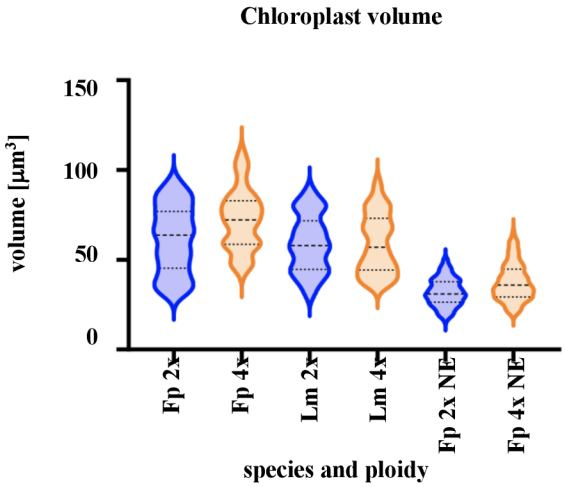

Figure 9: Visualization of the distribution of the obtained data for chloroplast volume

The difference between ploidy was observed for all analyzed parameters. The cell volume was lower in diploids and higher in tetraploids. In *L. multiflorum*, the difference was about 25.6% and in *F. pratensis*, diploids and tetraploids differ in cell volume by 34.6%. The difference was smaller (21.1%) in newly synthesized tetraploids and their diploid parental plants (Figure 6). The nuclear volume, on average, was always lower in diploids compare to tetraploids. The difference was 71.0% in *L. multiflorum*, 67.4% in *F. pratensis* and 56.3% between newly synthesized tetraploid *F. pratensis* and their diploid parents (Figure 7). Difference between ploidy in chloroplast number was 24.2% in *L. multiflorum*, 34.6% in *F. pratensis* and 15.4% between newly synthesized tetraploid *F. pratensis* and their diploid parents (Figure 8). The chloroplast volume differed between ploidies by 10.0% in *L. multiflorum*, 4.3% in *F. pratensis* and 17.5% between newly synthesized tetraploid *F. pratensis* and their diploid parents (Figure 9).

Second analyzed set composed allopolyploids and homoploid hybrids and their parents. This set contained 14 plants, five homoploid *L. multiflorum* × *F. pratensis* hybrids and their diploid parents, three *F. pratensis* × *L. multiflorum* and two *L. multiflorum* × *F. pratensis* reciprocal allotetraploid hybrids and their autotetraploid parents. 60 cells per plant were examined for chloroplast number and cell volume, 30 were examined to evaluate the volume of nuclei and chloroplasts. In this set, we focused on the potential heterosis in allopolyploids and homoploids. This was achieved by the comparison of values of the particular parameter of the allopolyploid (or homoploid) and the mid-parent value (MPV) calculated as an average from both parents. For cell volume, the heterosis was +34.7% in diploid *L. multiflorum* × *F. pratensis* hybrids, but -41.7% in *F. pratensis* × *L. multiflorum* and -96.4% in *L. multiflorum* × *F. pratensis* tetraploid hybrids (Figure 10). Hybrids (both diploids and tetraploids) had lower volumes of nuclei compare to their parents, with heterosis -11.5% in diploid hybrids, -35.0% in *F. pratensis* x *L. multiflorum* and -29.8% in *L. multiflorum* × *F. pratensis* tetraploid hybrids (Figure 11). Number of chloroplasts was higher in diploid hybrids compare to their parents, but lower in tetraploid hybrids compare to their parents. The heterosis was +17,4% in diploid hybrids, -10.1% in *F. pratensis* × *L. multiflorum* and -11.9% in *L. multiflorum* × *F. pratensis* tetraploid hybrids (Figure 12). In case of chloroplast volume, the hybrids had lower volume of chloroplasts than one of the parents (in diploid hybrids) or than both parents (in tetraploid hybrids). The heterosis was -4.9% in diploid hybrids, -28.9% in *F. pratensis* × *L. multiflorum* and -44.6% in *L. multiflorum* × *F. pratensis* tetraploid hybrids (Figure 13).

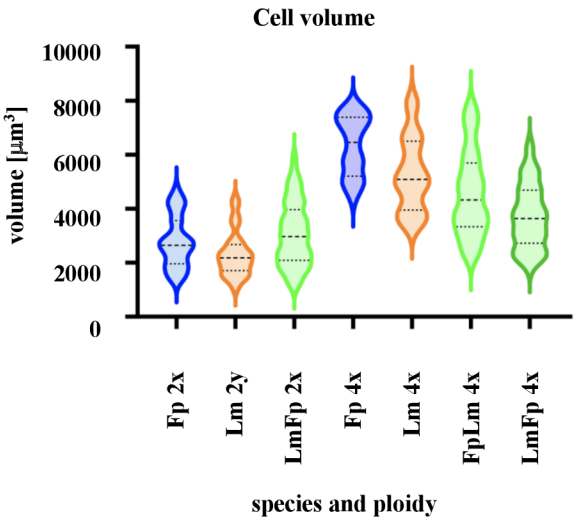

Figure 10: Visualization of the distribution of the obtained data for cell volume

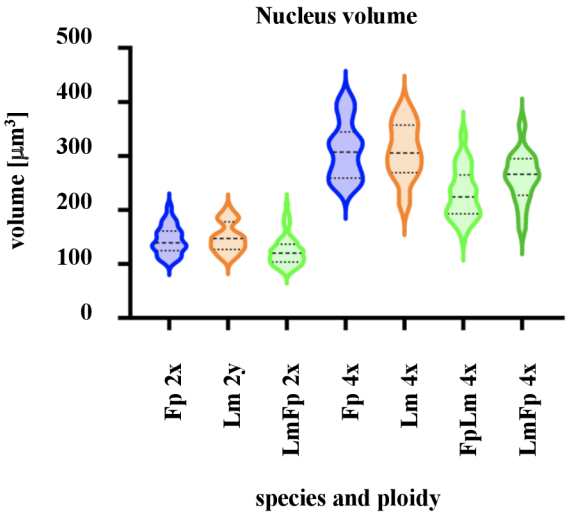

Figure 11: Visualization of the distribution of the obtained data for nucleus volume

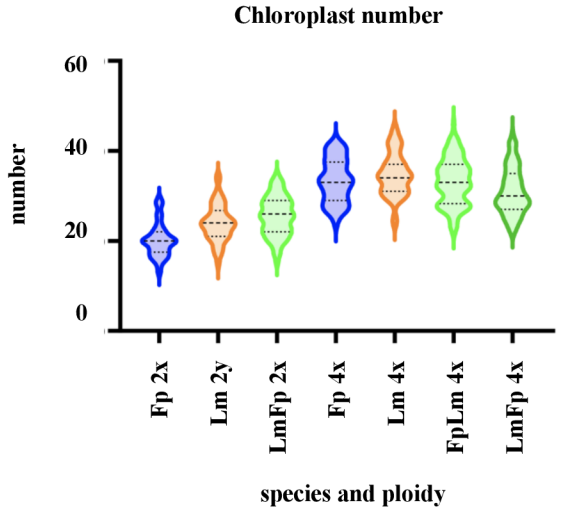

Figure 12: Visualization of the distribution of the obtained data for chloroplst number

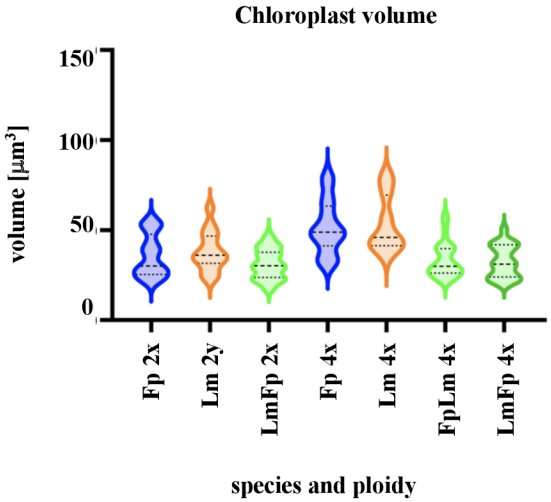

Figure 13: Visualization of the distribution of the obtained data for chloroplast volume

5.2 Determination of gene copy number in auto- and allopolyploids

In the second part of this bachelor thesis, the goal was to evaluate the copy number of genes involved in cytonuclear interactions in plant material as in the previous chapter: auto- and allopolyploids and their diploid counterparts (either parents or cultivars of the same species) using ddPCR.

5.2.1 Condition optimization

5.2.1.1 Primer annealing temperature

First step was to test oligonucleotide primers and then optimize the conditions. In this part were determined appropriate annealing temperatures for primers using gradient PCR. The temperature gradient consisted of steps 65, 64.5, 63.3, 61.4, 59, 57, 55.7, 55 ° C. In Figure 14 can be seen bands

formed in ideal range of temperatures for primers. For primer *rbcL* are optimal annealing temperatures between 59 and 55 °C, because there are no PCR products for temperature higher than 59°C. For the primer *psbD* is the temperature wider, it is between 63-55°C. Optimal annealing temperature range for primer *atpI* is same as for primer *psbD*. Range of temperatures for primer *atpA* is 61-55°C, however band for temperature 61°C is not so distinctive and was therefore not included. For primer *ndhC* are optimal annealing temperatures between 61 and 55 °C. For primer *atpC1* was determined a range between 61 and 55°C. Optimal annealing temperature range for primers *psbQ1, rbcS, cox2* and *cox15* are in whole range of used temperature gradient, which is 55-65°C. Range of temperatures for primer *ndhL* is 65-56°C, from whole temperature gradient this primer does not work only in the lowest temperature. Similarly for *nad6*, which works between 64 to 55°C, therefore does not work only in the highest temperature from used gradient. Optimal annealing temperature ranges for primers *nad7* and *matK* were determined to be 59-55°C. For primer *matR* is annealing temperature range between 61 and 55°C. Primer *actin* worked in range 63-55°C. Optimal annealing temperature ranges for all used primers are summarized in Table 14.

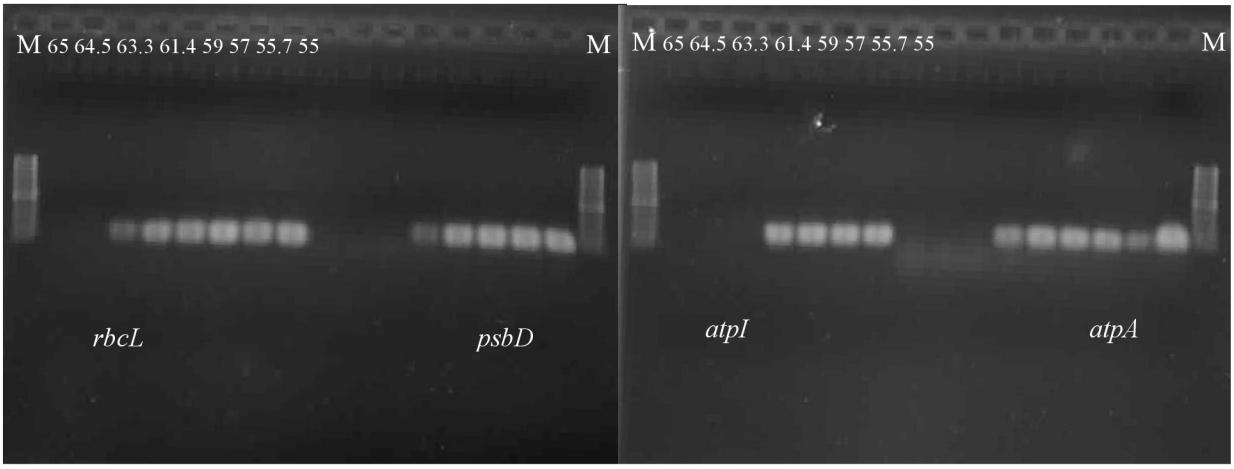

Figure 14: Visualization of PCR products on agarose gel; M – molecular weight standard, *rbcL* – primer *rbcL*, *psbD* – primer , *psbD*, *atpI* – primer *atpI*, *atpA* – primer *atpA*

Table 14: Optimal temperatures for primers

Gene	Temp [°C]
atpI	63-55
atpA	59-55
psbD	63-55
rbcL	59-55
ndhC	59-55
atpC1	61-55
psbQ1	65-55
rbcS	65-55
ndhL	65-56

cox2	65-55
nad6	64-55
cox15	65-55
nad7	59-55
matK	59-55
matR	61-55
actin	63-55

5.2.1.2 Determination of probe annealing temperature

The right annealing temperatures were determined using gradient ddPCR. Fluorescence amplitude js rendered against annealing temperature gradient. The pink line is the threshold, above which are positive droplets (blue/green) with PCR amplification and below are negative droplets (gray) without any amplification. The blue signal referes to probe for *Lolium multiflorum* gene and the green signal referes for *Festuca pratensis* probe. Eight ddPCR reactions are divided by the vertical dashed yellow line. Primer annealing temperatures were used as determined in the previous experiment (Table 14).

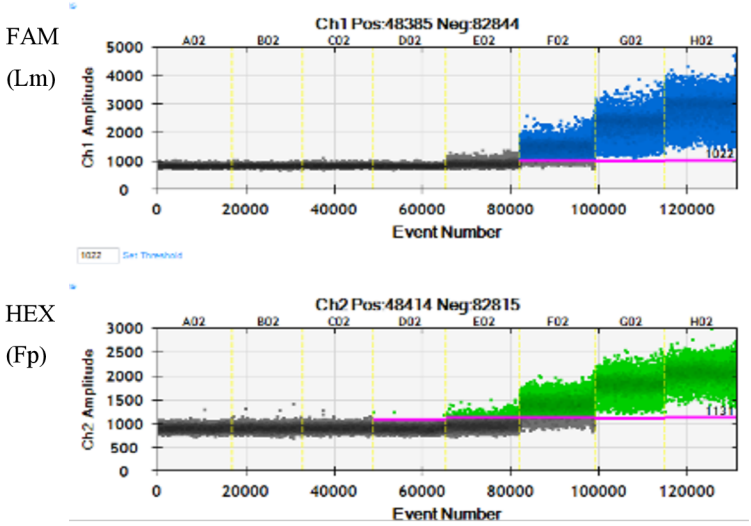

Figure 15: Graph visualizating number of positive droplets while thermal gradient optimizatio using *atpI* probe.

In Figure 15 we can see that the positive droplets are starting to occur at 61.4°C. 59°C was determined as optimal annealing temperature for used probe (*atpI*). For probe *atpA* was used gradient 63-55°C, optimal annealing temperature is 56°C. Temperature gradient for probe *atpC1* was 61-55°C, optimal annealing temperature is 58°C. Optimal annealing temperature for psbD probe is 57°C, determined using temperature gradient between 63 and 55°C. *rbcL* probe optimal annealing temperature is 53°C using gradient from 59 to 53. Temperature gradient for probe *rbcS1* was 65-55°C, optimal

temperature was determined to be 60°C. The optimal annealing temperatures are summarized in Table 15.

Table 15: Optimal temperatures for probes

Gene	Temp [C°]
atpI	59
atpA	56
atpC1	58
psbD	57
psbQ1	59
rbcL	53
rbcS1	60

5.2.1.3 Determination of appropriate template DNA and probe concentrations

Proper annealing temperatures were determined. However, it is obvious from Figure 16 that with the increased amplification rate the negative droplet population disappeared. This is probably caused by oversaturation of the reaction which results in all the droplets being positive. To avoid this issue, the concentration gradient of the template DNA and the probes respectively was used to find optimal concentrations. DNA concentration gradient was performed with probe *rbcL*. DNA of hybrid 8/2 (Table 7) and of diploid parents (Table 7) were used. In Figure 17 can be seen that the negative droplets started to appear when the DNA concentration was 0.1 $ng \cdot \mu l^{-1}$.

Next, to gain better separation of positive and negative droplet populations, the probe concentration gradient was performed. In Figure 18 can be seen that the best separation of positive and negative droplets occurred with half concentration used, which is 125 $ng \cdot \mu l^{-1}$. To be sure if there are not any extraneous nucleic acid contamination were used no template control (NTC). Single probe per reaction was used to check for potential cross-reactivity of the probes. Indeed, both probes were shown to react with its counterpart DNA. I.e. FAM probe designed to anneal only to *L. multiflorum* allele gave signal even in the presence of *F. pratensis* DNA alone and vice versa, HEX probe that should anneal selectively to *F. pratensis* DNA resulted in a strong signal in reactions containing only *L. multiflorum* DNA.

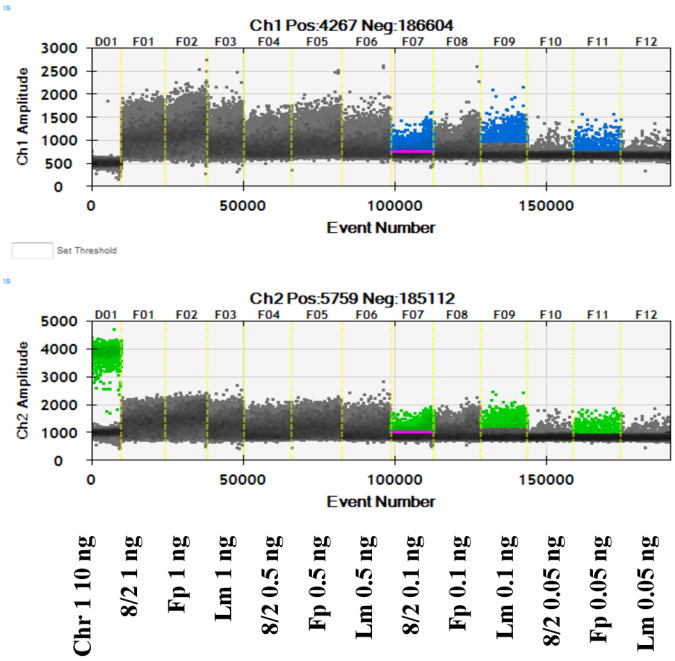

Figure 16: ddPCR plot showing the use of a concentration gradient to determine the ideal working concentration of the template DNA, probe *rbcL* was used for this experiment

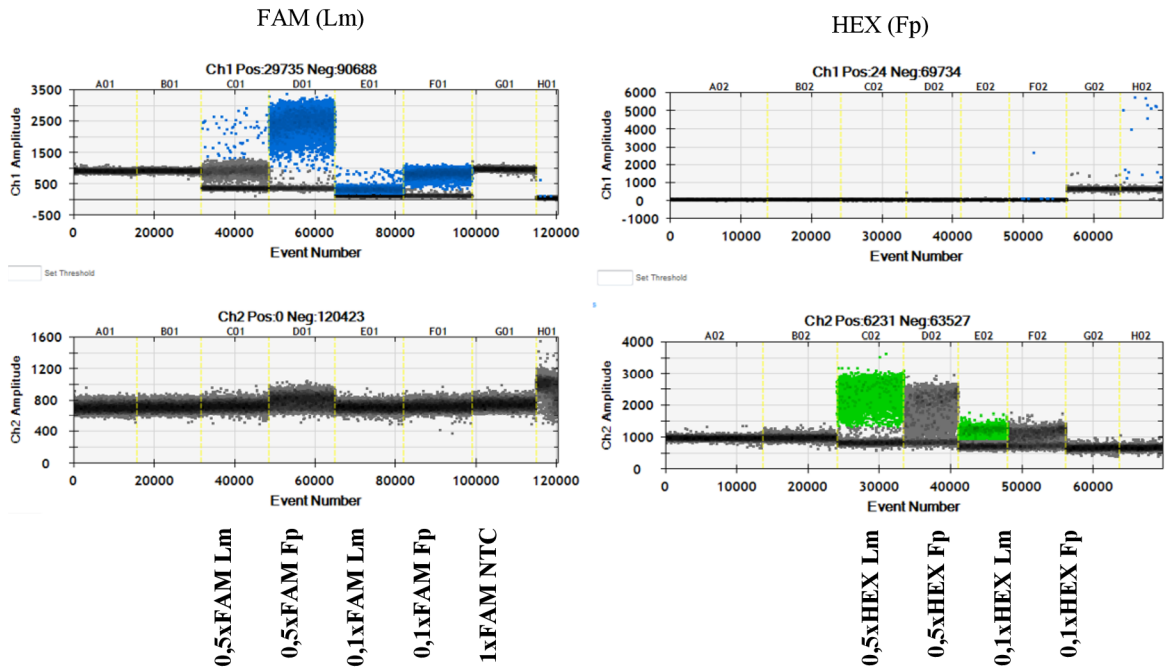

Figure 17: ddPCR plot showing showing the use of a concentration gradient to determine the ideal working concentration of the probe, probe *atpI* was used for this experiment

5.2.1.4 Digested DNA

To solve the problem with the cross-reactivity, DNA was cut with restriction enzymes HindIII and BsmI. These two enzymes were chosen because they do not digest the DNA at the sites of the selected genes. The digest was used to make the genes of interested more accessible to probe attachment. Used DNA was from diploid parents and from hybrid 8/2 (Table 7). After using the digested DNA, the cross reaction with the *Festuca* (HEX) probe disappeared, the cross reaction with the *Lolium* (FAM) probe remained (Figure 19). However, the separation of the positive droplets decreased. To reduce this problem, a further DNA or probe concentration reduction has been proposed. We have not been able to take this step due to a malfunction in the device.

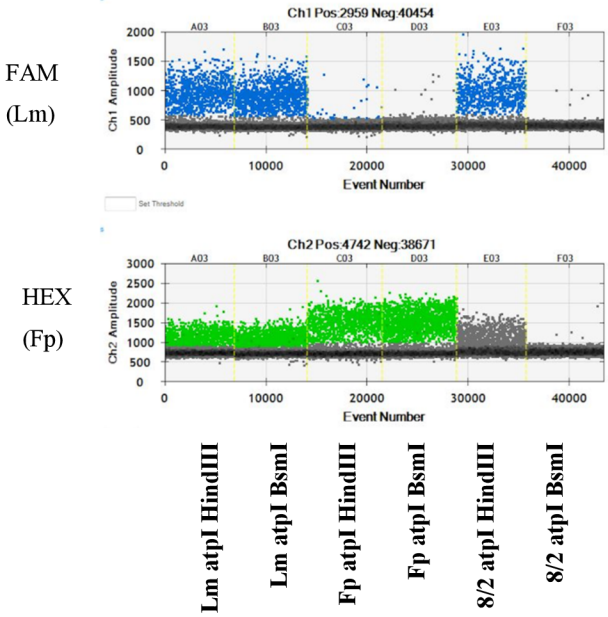

Figure 18: ddPCR plot showing showing the use of a digested DNA and its impact on preveting cross reactions

6 Discussion

Whole genome duplication refers to doubling the DNA content in a nucleus. This may cause the need for massive changes in genome and nuclear architecture. Our results revealed that volume of nucleus is for about 70% (71.0% in *L. multiflorum* and 67.4% in *F. pratensis*) higher in polyploids than in diploids. However, this increase is probably gradual over the generations, because newly synthesized autotetraploids of *F. pratensis* had only 56.3% increase. The increase of the cell size in polyploids is much lower compared to the increase of nuclear volume. We observed that polyploid cells are larger by 25.6% in *L. multiflorum* and 34.6% in *F. pratensis* than the cells of diploids. Similarly, to nuclear volume, the increase is also probably gradual. In newly synthesized tetraploid *F. pratensis*, there was only +21.1% increase. This correlates with studies of Butterfass (1991) who studied this changes in number of plants, he observed that the differences between volumes of cells was with increased ploidy 38 to 46%. Thus, it is evident that polyploid cell has to manage to organize the architecture of its nucleus with doubled volume of chromatin as well as the architecture of the cells with enlarged nuclei.

The other consequence of the polyploidization is the disturbed stoichiometry in cytonuclear interactions. Several protein complexes are composed of the products of nucleus-encoded and organelle-encoded genes. The text-book example is RubisCo, composed of large subunit encoded by nuclear gene *rbcL* and small subunit encoded by plastid gene *rbcS* (Andersson and Backlund, 2008). If the whole genome duplication appears, then the copies of nucleus-encoded genes in cytonuclear complexes are doubled without instant doubling the copies of the organelle-encoded genes. The polyploid can adopt various strategies to overcome this imbalance. One of them is to increase number of organelles. This study revealed that there is only small increase in the number of chloroplasts per cell in newly synthesized polyploids (15.4%), however, the number of chloroplast presumably increase in subsequent generations (up to 33.5% in polyploid cultivars, being in about F_7-F_8 generation). Such increase is relatively low compared to other studies. Tetraploid accessions of *Triticum monococcum* and *Aegilops tauschii* had 1.5 to 1.6 times more chloroplasts in mesophyll cells than their diploid counterparts (Pyke et al., 1987). The same was observed in sugar beet, where chromosome doubling increases the chloroplast numbers by about 72% (Mochizuki et al., 1955). Similarly, a survey of recent autopolyploids (130 entries) and induced allopolyploids (16 entries) showed an increase in chloroplast numbers in guard cells by 69% and by 67%, respectively (Butterfass, 1979). Thus, it is evident that increased number of chloroplasts is one, but not the only strategy used by polyploids to mitigate the effect of genome doubling on the well-tuned stoichiometry of cytonuclear interactions.

The other strategy involves the increase in the copy of organellar genomes in each organelle. I aimed to do such analysis, however, this research was not completed due to the malfunction of the droplet generator used for my study. Thus, I can only indirectly and very roughly estimate the increase in the copy number of plastid genomes based on the increase in the volume of chloroplasts.

In newly synthesized tetraploid *F. pratensis*, the chloroplast volume increased by 17.5%. However, the difference in chloroplast volume between diploids and tetraploids (well-established cultivars) was only 10% in *L. multiflorum* and 4.3% in *F. pratensis*. Data for the two subunits of RuBisCO indicate that an increase of copy numbers in the nuclear-encoded genes caused by polyploidization is not always fully compensated by an increase in copy number on the plastid side (Oberprieler et al., 2019). Similarly, in autotetraploid Arabidopsis thaliana, the copy number of chloroplast genes dropped slightly per nuclear genome (0.76-fold) compared to diploids, whereas the copy number of mitochondrial genes doubled (even though it was not clear whether this was due to an increase in the number of mitochondria per cell or via increased copy number of mtDNA molecules per mitochondria (Coate et al. 2020). The other strategies involve the increased expression of the organellar genes or decreased expression of nuclear genes and I aim to investigate these processes in my future work.

7 Conclusion

The goal of the first part of this bachelor thesis was to determine intracellular changes after polyploidization in autopolyploids and allopolyploids of *Festuca pratensis* Huds., *Lolium multiflorum,* Lam. and their hybrids (*Festulolium*). For visualization of these changes mesophyll cells were scanned using a confocal microscope and evaluated in Imaris software. I revealed that polyploidization leads immediatelly to the increase in the nuclear volume and less-pronounced increase in the cell volume. These changes are not one-step processes, but are ongoing in the successive generations. My thesis further revealed that the strategies to mitigate the disturbance of well-tuned stoichiometry of cytonuclear interactions in polyploids involve the increase of the number of chloroplasts and also their volume. Unfortunately, the exact copy numbers of genes involved in cytonuclear interactions have not been revealed due to the technical difficulties. However, the initial optimisation of ddPCR, a method for gene copy number evaluation, undertaken in this project, represents a significant step forward towards completing such measurements in the future.

Understanding of cytonuclear interactions and their changes following polyploidization can help us to assess their role in plant speciation and importance of polyploidization in plant evolution.

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