**Palacky University, Olomouc**

# Diploma thesis

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# Interactions between nuclear and cytoplasmic genome in auto- and allopolyploids

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**Summary:** Cytonuclear interactions are defined as interactions between the nuclear and cytoplasmic genomes, which result in the formation of several essential protein complexes, such as RuBisCo. However, whole genome duplication (WGD) can potentially lead to significant challenges for the maintenance of cytonuclear stoichiometry. The objective of this diploma thesis was to evaluate the changes that occur following the WGD in intracellular morphology, the number of chloroplast genomes, and the change in expression of a set of genes involved in cytonuclear interactions. The leaf mesophyll cells were observed in a confocal microscope and subsequently evaluated in IMARIS software. The evaluated traits were cell volume, nucleus volume, and chloroplast number and volume. The findings demonstrated that the doubling of the nuclear volume is accompanied by an increase in both the volume of the cell and the nucleus, accompanied by an increase in the number of chloroplasts, which is further pronounced in subsequent generations. The potential increase in the number of chloroplast genomes was estimated using digital droplet PCR (ddPCR). There was a significant rise in the number of chloroplast genomes, and this, together with the increase in the number of chloroplasts, effectively compensated for the doubling of the nuclear genome. Furthermore, alterations in nuclear and organelle gene expression were negligible in pure lines. However, there was a significant down-regulation of nuclear genes in established polyploids and a significant up-regulation of chloroplast genes in hybrids. The results of this work will contribute to our knowledge of how polyploidy affects plant systems. This topic is of interest not only the basic researchers but also to breeders to make informed choices when using polyploidization in the crop improvement.

## Bibliografické údaje

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**Shrnutí:** Cytonukleární interakce jsou definovány jako interakce mezi jaderným a cytoplazmatickým genomem, které vedou k tvorbě několika základních proteinových komplexů, jako je RuBisCo. Duplikace celého genomu (WGD) však může potenciálně vést k významným problémům při udržování cytojaderné stechiometrie. Cílem této diplomové práce bylo zhodnotit změny, ke kterým dochází po WGD v intracelulární morfologii, počtu chloroplastových genomů a změně exprese souboru genů zapojených do cytojaderných interakcí. Buňky mezofylu listů byly pozorovány v konfokálním mikroskopu a následně vyhodnoceny v softwaru IMARIS. Hodnocenými znaky byly objem buňky, objem jádra a počet a objem chloroplastů. Výsledky ukázaly, že zdvojnásobení jaderného genomu je doprovázeno nárůstem objemu buňky i jádra a nárůstem počtu chloroplastů, který se v dalších generacích dále zvětšuje. Potenciální nárůst počtu chloroplastových genomů byl vyhodnocen pomocí droplet digital PCR (ddPCR). Došlo k výraznému nárůstu počtu chloroplastových genomů, který spolu s nárůstem počtu chloroplastů účinně kompenzoval zdvojnásobení jaderného genomu. Změny v expresi jaderných a organelových genů byly ve většině případů zanedbatelné. U zavedených polyploidů však došlo k výraznému snížení transkripce jaderných genů a u hybridů k výraznému zvýšení transkripce chloroplastových genů. Výsledky této práce přispějí k našim znalostem o vlivu polyploidie na rostlinné systémy. Toto téma je zajímavé nejen pro základní výzkumníky, ale také pro šlechtitele, aby se mohli informovaně rozhodovat při využívání polyploidizace při zlepšování plodin.

## Statement

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

In Olomouc…… ……*Kubíková*……

## Acknowledgment

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# 1 Introduction

Polyploidization is defined as the acquisition of more than two chromosome sets. Polyploidy is recognized as a significant driving force behind plant evolution (Ramsey and Schemske, 1998). It was first identified more than a century ago (Strasburger, 1910) and it is a natural phenomenon occurring in numerous eucaryotic taxa. Whole genome duplication (WGD) represents a fundamental mechanism underlying numerous major episodes of diversification in land plants (Van De Peer et al., 2017) and remains a prominent process in plant speciation (Barker et al., 2016). Furthermore, it is an important tool in the plant breeding to stabilize the genome and overcome bottlenecks linked to interspecific hybridization.

The plant cell contains three distinct compartments where DNA is stored: the nucleus, mitochondria, and plastids. Consequently, newly developed polyploids are confronted with the challenge of maintaining the precise stoichiometry between nuclear-encoded and organelle-encoded genes engaged in cytonuclear interactions, such as Rubisco, whose large subunit is encoded by the chloroplast *rbcL* gene and the small subunit, which is encoded by the nuclear gene *rbcS*. As illustrated by Sharbrough et al. (2017), the doubling of copy numbers of nuclear genes following WGD does not result in the duplication of the number of organelles. One indicator that polyploidy has considerable stoichiometric implications in the context of cytonuclear interactions is that organelle-targeted genes appear to be one of the earliest and most common classes of nuclear genes to revert to a single copy following an event of WGD. Furthermore, the well-documented positive correlation between nuclear genome size and cell size (Beaulieu et al., 2008) indicates that plants may be capable of storing a greater number of organelles per cell as nuclear genome copy number and cell size increase, exemplified by the observation that alfalfa (*Medicago sativa*) polyploids exhibit elevated levels of chloroplast numbers per cell compared to diploids (Bingham, 1968).

A significant challenge can arise in allopolyploids and interspecific hybrids concerning genes involved in cytonuclear interactions. The typical inheritance of the cytoplasmic genomes is uniparental (usually maternal), while the nuclear-encoded genes originate from both parents. These genes may be diversified between parental genomes and are not interchangeable (Sharbrough et al., 2017). The cytonuclear interaction has been a subject of interest to plant scientists for almost two decades, during which time

various databases have been created to depict genes involved in the cytonuclear interaction. The most recent of these is the CyMIRA database, which was developed as a Cytonuclear Molecular Interactions Reference for Arabidopsis and includes 910 nuclear-encoded genes involved in direct cytonuclear molecular interactions (i.e. components of cytonuclear enzyme complexes) (Forsythe et al., 2019). The databases will facilitate further studies aimed at elucidating the biological mechanisms underlying the reestablishment of stoichiometry between cytoplasmic and nuclear genes following polyploidization. Such studies will enhance our understanding of the evolutionary significance of polyploidization, which represents a principal mechanism of plant speciation.

My diploma thesis is focused on changes in cytonuclear interactions in neopolyploids and established polyploids in two plant groups: Festuca-Lolium group (*F. pratensis* and *L. multiflorum*) and *Arabidopsis* group (*A. thalina, A. lyrata* and their hybrids). The objective of this thesis is to ascertain whether there are any discernible alterations in plant morphology that could potentially influence cytonuclear interactions, and to examine how an individual responds to the significant changes that follow the WGD.

# 2 The work goal

1. Literature research on the topic of polyploidy and cytonuclear interactions.
2. Microscopic analyses of plant cells in diploid and polyploid individuals (*Arabidopsis thaliana* and *A. lyrata*) 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.
4. Determination of changes in gene expression following polyploidization for the selected set of genes involved in cytonuclear interactions using qPCR.

# 3 Literary review

Polyploidy is defined as the condition where an organism possesses three or more sets of chromosomes. This stable condition can be inherited by offspring. Many polyploid species are well adapted to their environment, and it is believed that diploid species such as humans and *Arabidopsis* evolved from polyploid ancestors (Van de Peer and Meyer, 2009).

However, polyploidization leads to significant changes in genome structure and nuclear organization, which raises questions about cell cycle processes, cell physiology, gene expression regulation, and genome stability. In plants, polyploidy is a common phenomenon, particularly in the context of interspecific hybridization, which frequently results in whole genome duplication and subsequent stabilization of hybrid genomes through diploidization. Species that have undergone polyploidization and diploidization are referred to as paleopolyploids (K. Zhang et al., 2019).

Polyploidy is less prevalent in animals, although some polyploid species can be found among frogs (Schmid et al., 2015), fish (Zhou & Gui, 2017), insects (Z. Li et al., 2018), and mammals (Acharya & Ghosh, 2016). It is noteworthy that even diploid organisms can exhibit polyploid tissues, as an example of this are hepatocytes in the human liver.

## 3.1 Polyploidy and its effects

Polyploidy is defined as a condition in which the genome of an organism consists of three or more sets of chromosomes. Polyploidy is a direct consequence of whole genome duplication (WGD). It is a feature common to most basic land plant lineages and an important evolutionary force. The prevalence of polyploidy varies between different eukaryotic lineages but is particularly widespread in the plant kingdom (Otto and Whitton, 2000). Polyploidy can confer many advantages, such as increased resistance to the environment, but also disadvantages, such as meiotic instability (Comai, 2005). Polyploidy is often considered to drive plant speciation, although the mechanism of postzygotic barriers between ploidy levels can be impermeable. Polyploid lineages can contribute to the exchange of genes between levels and recently emerged polyploids have to overcome the disadvantage of being a minority and adapt to new niches (Tayalé & Parisod, 2013). Despite these disadvantages, polyploidy is still widespread, and many species that are currently diploid show evidence of polyploid ancestry (Van De Peer et

al., 2021).

These multiplied sets of chromosomes coexist in a single nucleus and are inherited by offspring. Two different types of polyploids are usually considered: autopolyploids, with extra sets of chromosomes derived from a single species, and allopolyploids, with sets of chromosomes derived from different species as a result of interspecific hybridization (Comai, 2005).

During evolution, duplicated genes underwent a process of diploidization (Figure 1). This process involves the loss or severe alteration of many duplicated genes and the rearrangement and reduction of the chromosomes (Hollister, 2015). There are three possible outcomes of duplicated gene evolution: degenerative mutations, neofunctionalization, or sub-functionalization. Most mutations are deleterious, and therefore one copy of a gene is expected to be lost relatively quickly, except in populations of large effective size (Lynch & Conery, 2000). It is assumed that WGD might reduce the probability of extinction and increase the chance of surviving (Donoghue & Purnell, 2005; Fawcett et al., 2009). WGD is involved in environmental adaptation and speciation. In some cases, polyploids live in broader or different habitats

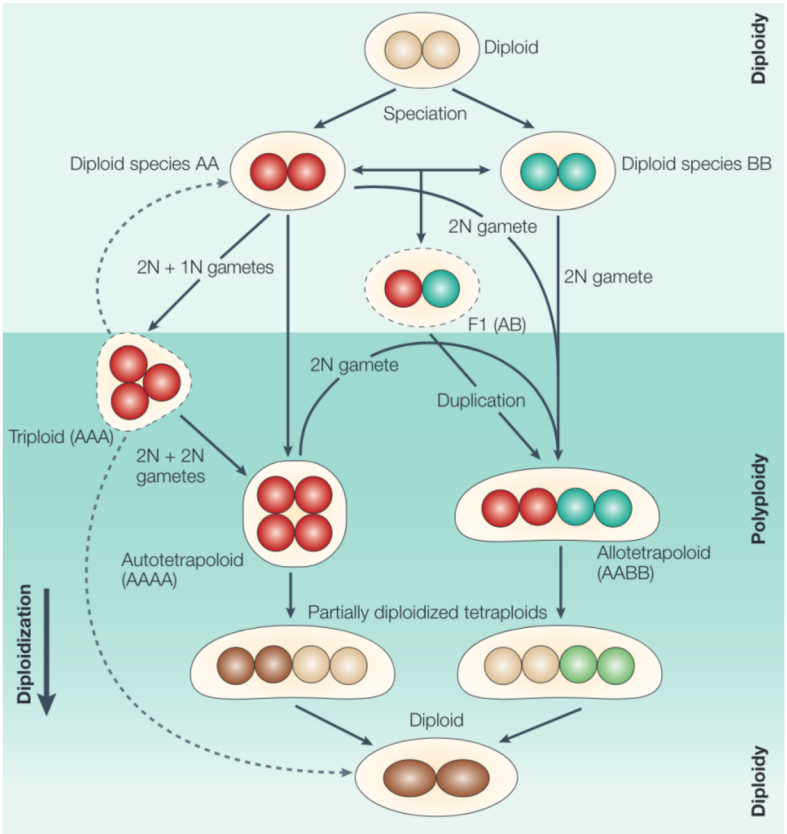



**Figure 1:** Illustration showing the possible paths leading to polyploidy and the gradual transition back to the diploid state (Adopted from Comai, 2005).

than their diploid ancestors (Parisod et al., 2010). Geographical differences in the frequency of polyploids between plants were also observed, they were influenced by climate and the composition of flowering plants (Rice et al., 2019). Polyploidy has been associated with stress tolerance, larger fruit seeds, or variation in metabolite content (Renny-Byfield & Wendel, 2014), making it interesting for agricultural use. It has been successfully used to increase yield and biomass in several crop species such as potato or watermelon (Sattler et al., 2016).

Response to WGD can be observed in several traits such as anatomical and biochemical changes which have an impact on physiological processes in plants. The most frequent phenotype which is associated with polyploidy is nucleus and cell enlargement and change in the number of organelles. These changes affect the rate of carbon dioxide diffusion, as well as intra- and intercellular biochemistry and metabolism. Other effects include changes in gene product levels (polyploidy dosage effects) and biochemical diversity (produced by new allelic combinations in polyploid plants) (Warner & Edwards, 1993). However, there are differences both between and within species, and in particular between the types of cells within an organism (Doyle & Coate, 2019).

It takes many generations for duplicate genomes to evolve. However, to understand the short-term consequences of recently evolved polyploidy (neopolyploidy) for the fitness and adaptation of organisms is also important. There is growing evidence that neopolyploids have a higher uptake of ions and are more tolerant to salt and drought than diploids in the same genetic background (Allario et al., 2013; Chao et al., 2013). Thus, the physiological correlation of WGD may help to facilitate the invasion of new or challenging environments (Stebbins, 1985). Polyploidy can also increase the rate of adaptation within populations under certain conditions (Otto & Whitton, 2000). The challenges of neopolyploids have received less attention. Neopolyploids often show major changes in gene expression patterns, alterations in the epigenetic regulation of transposable elements, and mitotic and meiotic problems (Comai, 2005). Successful polyploid lineages must overcome these early challenges because they are important cellular processes and have a strong impact on fitness.

### 3.1.1 Anatomical effects of polyploidy

The enlargement of the cells has a global effect on the entire organism that has

undergone WGD. One of the main cell types affected are guard cells, epidermal cells that control stomata. Guard cells tend to be larger in both new and evolved polyploids compared to the diploids but are observed to decrease in size over time in more established polyploids (Butterfass, 1987).

Stomata size and density have a significant impact on the rate of $CO_2$ uptake and water transpiration in plants (Brodribb et al., 2020). Research on *Arabidopsis thaliana* has shown that mutants with higher stomatal density exhibit increased $CO_2$ assimilation under high-light conditions. In contrast, those with reduced stomatal density have lower gas exchange and photosynthetic rates (Schluter, 2003). However, the effect of the stomata size and density on polyploid plants, which have larger stomata and lower density, remains unclear. The outcome is determined by the rate and extent of stomatal response to environmental or hormonal stimuli. Polyploids have been observed to exhibit both lower and higher rates of gas exchange, $CO_2$ assimilation, and photosynthesis (Bomblies, 2020).

Polyploidy typically results in a reduced growth rate, except in neopolyploids where compensation can occur (Gupta, 1981). The reasons for slower growth include changes in metabolism and hormone levels, such as decreased auxin and brassinolide levels (Ma et al., 2016; C. Zhang et al., 2020). However, in some cases, polyploidy may increase the expression of genes related to cell wall formation, leading to accelerated growth. The mechanisms behind polyploidy's effect on growth rate remain unclear (Robinson et al., 2018).

In neotetraploid and evolved tetraploid *Chamerion angustifolium*, vascular elements are wider than in diploids, resulting in higher hydraulic conductivity. However, tetraploids show no difference in vulnerability to cavitation. Tetraploids may further increase conductivity in response to changes in water conditions caused by polyploidy (Maherali et al., 2009). Autotetraploid *Atriplex canescens* also have broader xylem elements, but lower hydraulic conductivity than diploids under non-stressed conditions. However, they are more resistant to drought-induced conductivity loss and can maintain higher $CO_2$ assimilation and photosynthesis under heat- and water-limited conditions (Hao et al., 2013). Polyploid plants in stressful environments have higher vessel diameters and resistance to drought-induced embolism due to differences in vascular architecture (Maherali et al., 2009). Polyploid plants may modify their xylem "safety elements" to reduce the risk of cavitation. Plastic adaptive responses in the xylem can mitigate this risk, such as increasing resistance to cavitation with height and modifying growth with

climate change (Burgess et al., 2006).

Cell enlargement can also have an impact on biomass increase. The polyploid individuals show enlargement of organs such as roots, leaves, flowers, or seeds compared to their diploid ancestors (Sattler et al., 2016). This has been implemented in plant breeding programs to increase the production of some agricultural crops such as cotton, wheat, banana, and watermelon (Sattler et al., 2016). However, the increase caused by polyploidy is neither linear nor constant (Tsukaya, 2013). Experiments on *A. thaliana* have shown that octaploid plants are much smaller than tetraploid or even diploid plants, suggesting that body size is not passively regulated by ploidy level (Corneillie et al., 2019; Tsukaya, 2013).

Cell volume and nuclear volume are closely correlated, with both increasing during the cell cycle in human and yeast cells. In plants, nuclear size is linearly related to cell volume. Transport from the cytoplasm plays a role in determining nuclear size (Doyle & Coate, 2019). It was concluded that nuclear size control is central to the proper regulation of cytoplasmic and nuclear membrane transport (Kume et al., 2017).

The study concluded that the more DNA a set of chromosomes contains, the more chloroplasts are usually found in the cells (Butterfass, 1987). Another study also showed that DNA content, chloroplast number, and photosynthetic rate increase with ploidy (Warner & Edwards, 1993). However, the increased number of chloroplasts can bear some functional consequences, such as changes in photosynthesis (Doyle & Coate, 2019).

### 3.1.2 Biochemical effects of polyploidy

As stated above, polyploidy may cause an increase in organ size, growth vigor, adaptability, and stress tolerance compared to diploid plants. Enlargement of the leaves can improve their ability to capture light and $CO_2$, thus contributing to an improvement in photosynthesis (Evans, 1999). At the cellular level, photosynthetic efficiency increases with higher DNA content per cell, cell size, and photosynthetic pigment content. Polyploid plants often have larger and denser chloroplasts, which is advantageous for photosynthesis. However, many wild tetraploid species do not exhibit significantly higher photosynthetic efficiency than diploid species at the organ level. This phenomenon may be due to a reduction in the number of cells per unit area after polyploidization.

Photosynthetic capacity is a significant factor in the success of polyploids. Anatomical changes include alterations in cell size and leaf thickness, which affect the

rate of $CO_2$ diffusion. Biochemical changes include an increase in the amount of photosynthetic proteins and enzymes synthesized. Most of these traits exhibited higher values in polyploids than in diploid progeny (Warner & Edwards, 1993). However, the photosynthetic rates expressed per unit leaf area varied among polyploids. The effect of polyploidy on photosynthesis varies with the increasing complexity of the trait of interest.

Genetic changes following WGD can include modifications in the regulation of gene expression, loss of genes, emergence of new functions, and changes in gene balance. In some aromatic and medicinal plants, individuals with a higher number of complete sets of chromosomes have been observed to produce a greater amount of secondary metabolites (Iannicelli et al., 2020). However, there are exceptions where polyploidization does not affect secondary metabolite production (Wohlmuth et al., 2005). Various plant species respond differently to polyploidization, indicating that ploidy level may affect the regulation of metabolite synthesis.

Polyploidization may impact enzyme activity per unit protein. Studies indicate that polyploids with increased RNA and genome copy number may experience changes in metabolite production. Analyses of the effect of polyploidization on the expression levels of genes involved in the synthesis of metabolites such as morphine, artemisinin, or vindoline (Mishra et al., 2010; Lin et al., 2011; Xing et al., 2011) confirmed that certain genes are overexpressed in polyploids, leading to increased metabolite production. However, it is not always the case that enzyme activity doubles when the genetic dose is doubled. Some enzymes are not affected or even have lower expression levels in polyploids. These findings indicate that polyploidization has differential effects on gene expression and enzyme activity (Iannicelli et al., 2020).

### 3.1.3 Stress resilience

The relationship between stress and polyploidization is well supported by studies of modern polyploids. There is a long history of polyploidization being associated with abiotic stress, particularly adaptation to dry and cold environments. Studies have shown that polyploids tend to occupy drier habitats than diploids in the same groups (Van De Peer et al., 2021). The frequency of polyploidy increases from the equator to the poles (Rice et al., 2019), and within the Arctic, higher-order polyploids are more common at higher latitudes (Brochmann et al., 2004). Genome duplication has been found to result in changes related to adaptation to drought stress, including alterations in transpiration,

water use efficiency, photosynthetic rate, phenology, antioxidant response, and morphology (Maherali et al., 2009). As an example, natural tetraploid *Arabidopsis thaliana* plants have increased salt tolerance compared to diploid plants (Chao et al., 2013). A higher level of tolerance to abiotic stress has also been demonstrated in polyploid fungi and animals. Experimental evolutionary studies in yeast (*Saccharomyces cerevisiae*) have shown that genome duplication can confer a fitness advantage during periods of stress (Selmecki et al., 2015). Another example is a frog species of the genus *Neobatrachus*. While the diploid species are geographically isolated, tetraploid species of the same genus are widely distributed throughout Australia. This shows that in the face of climate change, the adaptive advantage of tetraploids may be enhanced (Novikova et al., 2020). While physiological, cellular, and genomic responses to stress have been studied in polyploids, the molecular processes underlying these responses are not fully understood (Fox et al., 2020).

Stress-tolerant polyploids often show a similar type of stress response to diploids. These include changes in gas exchange, stomatal opening, chlorophyll content, photosynthesis, and water content in response to drought and salt stress but these changes are not as severe in polyploid plants (W.-D. Li et al., 2009; Van Laere et al., 2011; Wei et al., 2019). The differences between the diploids and the tetraploids can be attributed to differences in the anatomy of the leaves and/or the rate of transpiration. Research has shown that differences in transpiration rate, drought tolerance, and water use efficiency can be explained by stomatal size and density alone (Caine et al., 2019; Hughes et al., 2017). The size of the stomata and their density can also influence the salinity tolerance, probably by changing the water transport in the plant and thus the uptake of $Na^+$ ions. Salinity tolerance may also be related to the stomatal closing, as shown by the observation that diploid rice with higher closure and lower density of stomata is also very drought and salt-tolerant (Huang et al., 2009). Other studies suggest that polyploids are less susceptible to cellular stress and have greater reactive oxygen species (ROS) scavengers and antioxidant capacity, which may contribute to overall stress resistance. The reduced availability of resources under stressful conditions may also in some cases favor polyploids because they tend to grow more slowly, which means that they are less likely to suffer cell damage (Deng et al., 2012).

Biotic stress in plants is caused by pests, parasites, and pathogens such as bacteria, fungi, oomycetes, and nematodes. These organisms can cause various diseases and reduce crop yields. Pathogens such as biotrophic fungi can cause leaf spots and tumors, while

necrotrophic fungi destroy plant cells. Viral infections retard growth and cause plant deformities. Parasitic nematodes can damage the root system and transmit viruses. Pests can also reduce yields and transmit diseases (Schumann & D'Arcy, 2006). Polyploidization can affect plant resistance to pathogens and insects. There is an evidence that polyploid species may be more resistant than their diploid ancestors (Thompson et al., 1997).

## 3.2 Hybridization

Hybridization is the crossing of genetically distinct groups or taxa that results in the production of viable hybrids. These interactions can produce new phenotypes with different advantages and disadvantages, influencing speciation. Hybridization occurs in different spatial and temporal contexts, including border hybrid zones, interpopulation gene exchange, and habitat responses (Abbott et al., 2013).

Hybridization can be common, widespread, localized, or globally rare, and its consequences depend on population growth or shrinkage (Currat et al., 2008). It is a complex and ever-changing interaction that can take hundreds to millions of generations to evolve. The size and spatial distribution of populations change, and hybridization may occur at different stages or locations (Hewitt, 2011).

Hybrid speciation through changes in chromosome number and chromosomal rearrangements can lead to reproductive isolation and hybrid speciation (Schumer et al., 2014). Homoploid hybrid speciation appears to be more common than previously thought. There are examples of homoploid hybrid speciation in various animal and plant species (Mavárez & Linares, 2008). Some scientists believe that hybridization may have played a role in the origin of the species *Homo sapiens*, but evidence for this is lacking and many cases are still debated (Mallet, 2005).

In agriculture, breeders have focused on hybrid crops. Many crop species originated through interspecific or even intergenomic hybridization often followed by WGD. About 15% of crops have undergone ploidy shifts during domestication, similar to speciation events involving ploidy shifts (Warschefsky et al., 2014). For instance, domesticated crops such as wheat, tall fescue, cotton, banana, oilseed rape, apple, date palm, and citrus species are of hybrid origin (Kopecký et al., 2022).

Another focus of breeders is on hybridization between crops and their wild relatives. One aim is to introduce adaptive traits from wild relatives into cultivated forms

through breeding programs. Vavilov's (1926, 1951) research pioneered the concept of directed introgression, the transfer of adaptive traits from wild relatives to crop plants. This technique has found significant application in several annual crops such as wheat, rice, barley, cassava, potato, and tomato. Numerous studies have focused on the transfer of desirable traits from 185 wild relatives to 29 different crop species (Warschefsky et al., 2014). Genetic analyses show evidence of significant gene flow between crops and wild species in maize, barley, rice, and potato. Domestication and global spread of these crops brought them into contact with new populations of wild relatives, leading to adaptive introgression (Janzen et al., 2019).

### 3.3 The cytoplasmic genome

The cytoplasmic genome is represented by circular DNA molecules located in semiautonomous organelles, i.e. mitochondria and chloroplasts. The organelles originated by a process of endosymbiosis, mitochondria from free-living α-proteobacteria, whereas chloroplasts derived from free-living cyanobacteria (Rand et al., 2004). The first fully articulated version of the endosymbiotic theory was presented by Mereschkowsky's proposal in 1905 (Martin & Kowallik, 1999). The discovery that organelles have their own DNA at all was one of the key observations that supported endosymbiotic theory in the first place (Doolittle, 1980). This theory was finally confirmed through DNA and protein sequencing and phylogenetic analysis of these organelles. Our understanding of the origin of plastids has been enriched by genomics.

Currently, it is known that the organellar genome underwent extreme reduction through endosymbiotic gene transfer (EGT), leaving its imprints in the nuclear genome (Timmis et al., 2004). The loss of redundant or non-functional organelle genes, such as the "use-it-or-lose-it" case of reduced chloroplast genomes in non-photosynthetic plants (dePamphilis & Palmer, 1990) serves as an example of reciprocal co-evolution. On the other hand, the loss of genes that have been transferred to the nucleus does fit the definition of co-evolution (K. Adams & Palmer, 2003).

The transfer of genes from mitochondria to the nucleus is an important process that requires the incorporation of mitochondrial nucleic acids into the host chromosomal site and the acquisition of expression signals to drive transcription. It also requires the acquisition of sequences for targeting proteins to the mitochondria and the adaptation of the different genetic codes of organelle and nuclear genes. These gene transfers occur

frequently in gymnosperms (Rand et al., 2004). In the past, cytonuclear fusion has caused a large number of organelle-to-nucleus gene transfers, creating mutational pressure on the nuclear genome, and probably influencing natural selection. This gene transfer caused genetic changes that created new selective pressures (Timmis et al., 2004).

Experimental evolutionary approaches have allowed scientists to study the movement of organellar DNA into the nuclear genome and how genes are affected in the process (Stegemann & Bock, 2006). In plants, a significant proportion of the nuclear genome is dedicated to the control of both organelles. As part of this control, many proteins encoded by transferred genes are redirected to the plastid and mitochondrial compartments. These proteins include not only enzymes and structural components but also regulatory factors that respond to changes in the external environment and influence the expression of organellar genes. In addition to this nuclear control, signalling pathways originating in the organelles can also influence gene expression in the nuclear genome. The intricate network of anterograde (nuclear control of organellar functions) and retrograde (signalling pathways from organelles that influence nuclear genome expression) signalling relies on the close coordination between nuclear and organellar functions (Greiner & Bock, 2013; Pfannschmidt, 2010).

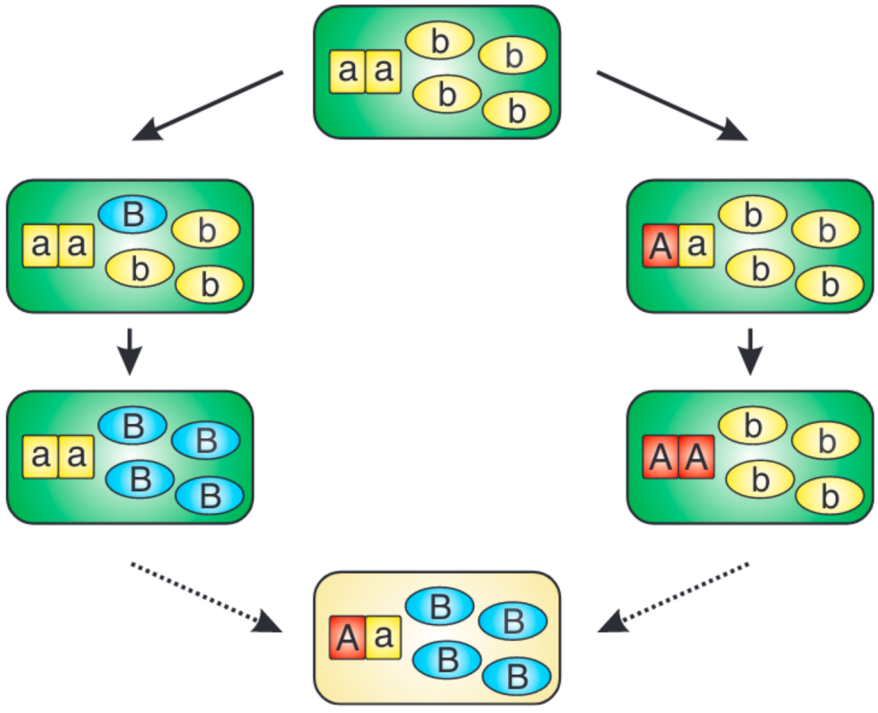



**Figure 2:** The Dobzhansky-Muller model, modified for nuclear and organelle coevolution, explains the formation of reproductive barriers in an ancestral population based on the interaction of genes a and b. Hybridization between subpopulations with different alleles leads to reduced fitness of the offspring and the formation of a postzygotic hybridization barrier (Adopted from Greiner et al., 2011).

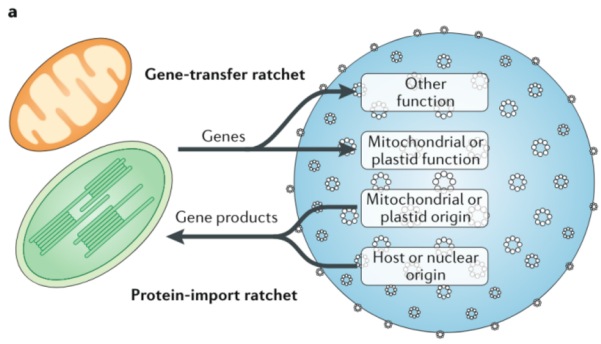
The terms genotype and plasmotype distinguish between genes in the nucleus and in organellar genomes. Genotype refers to the genetic information of the nucleus, while plasmotype includes information contained in the plastid and mitochondrial genomes. The genotype and plasmotype evolve by different mechanisms and at different rates. Nuclear genes and their alleles are constantly rearranged by sexual recombination, whereas organellar genes are almost unaffected by recombination. The separation of organelles is due to their predominantly uniparental inheritance and the absence of organelle fusion in plastids, which are typically passed down from the mother (Birky, 2001). In asexual reproduction, deleterious mutations can accumulate in the genome, a phenomenon known as Müllerian latch (Figure 2). However, the biparental inheritance of the chloroplast genome has been found in about 20% of naked-seeded plants, but the actual frequency remains uncertain (Barnard-Kubow et al., 2017). Biparental inheritance may have evolved as a mechanism to overcome incompatible plastids or to counteract deleterious mutations (Barnard-Kubow et al., 2017). Curiously, the rate of mutation in the genomes of the plastids and mitochondria in plants is significantly lower than in the nuclear genome (Wolfe et al., 1987). The mechanisms used by plastids and mitochondria to minimize mutations are not yet fully understood. The existence of multiple copies of organelle genomes and the ability to perform gene conversion contribute to keeping mutation rates low (Khakhlova & Bock, 2006).

The nuclear and organellar genomes are in close interaction, even though they are physically separate. Many large protein complexes exist that are composed of both organellar and nuclear genes. Therefore, these genes evolve together. Genetic changes are necessary to adapt to a new environment, and these changes occur in processes that are partly encoded by the organellar genome. These changes are likely to occur in coordination with the genotype and the plasmotype. For example, if adaptation involves optimizing photosynthetic electron transport under stress, mutations in both organellar and nuclear genes are likely to occur. This indicates that although the genomes are physically distinct, there is limited scope for independent evolution, and evolution occurs through the interdependence of these genomes (Greiner & Bock, 2013).

A model of coadaptation exists involving compensatory mutations between interacting genes (or the position of nucleotides within a gene). In this way, if one of the loci is mutated, the other will be mutated as well. Given Muller's ratchet environment of the endosymbiont genome, co-adaptation in the cytonuclear environment should be inherently asymmetric. Nuclear genes are more likely to contribute to the adaptive

compensatory mutations that maintain co-adapted states between interacting genomes because positive selection is facilitated by recombination and large effective population sizes (Rand et al., 2004).

As stated before, studies show that genes from organelle genomes have moved into the nucleus during evolution. Gene transfers have a significant impact on genome evolution and transgenic technology (Timmis et al., 2004). A comparison of the mitogenomes of different species showed that the loss of mitochondrial genes occurred in two phases (Janouškovec et al., 2017). The first phase occurred before the last common ancestor of eukaryotic organisms, while the second phase was heterogeneous and occurred in different lineages and at different times. The genes conserved in the organelle genomes focus on cellular respiration, photosynthesis, and the translation apparatus (Sloan et al., 2018).




b

| Subcellular localization or function | Original source of nuclear genes: Mitochondrial or plastid | Original source of nuclear genes: Host or nuclear |
|---|---|---|
| Mitochondrial or plastid | Gene transfer and return targeting to organelles<br>• Transferred OXPHOS or photosynthetic subunits<br>• Transferred organelle ribosome subunits | Novel recruitment and functional replacement in organelles<br>• Import machinery<br>• ATP–ADP translocases<br>• Imported tRNAs for organellar translation |
| Other | Novel recruitment and functional replacement in the host or nucleus<br>• Components of carbohydrate and nucleotide metabolic pathways | Retention of ancestral host or nuclear function<br>• Nuclear DNA replication and transcription machinery<br>• Cytosolic ribosomes |

**Figure 3:** Trends in cytonuclear movement of genes and gene products. a) Summary of movements of genes and gene products between nucleus and organelles. b) List of genes and their origin and location involved in cytonuclear interaction. (Adopted from Sloan et al. 2018).

Two asymmetries are associated with gene transfer between the nucleus and the organelles (Figure 3). First, gene transfer occurs mainly from the organelles to the nucleus (Timmis et al., 2004), and second, gene products are mainly transferred from the nucleus to the organelles (Paila et al., 2015). It is argued that the transfer of organelles into the nucleus and the subsequent replacement of existing functions by these genes is not unexpected, but rather a natural by-product of DNA movement (Doolittle, 1998). This process creates the opportunity for occasional insertions that can become functional replacements for existing nuclear genes.

### 3.4 Cytonuclear interactions

Genetic control in eukaryotes is divided between the nucleus and one or more genomes located in the cytoplasm. As discussed in the previous chapter, this is a consequence of the endosymbiotic origin of organelles (mitochondria and plastids) that

retain remnants of their ancestral genomes. This complex arrangement of genomic compartments has been maintained over billions of years of evolution (Greiner & Bock, 2013). The genetics of the nuclear and cytoplasmic genomes differ in many ways, such as genome copy numbers, mutation rates, modes of inheritance, and mechanisms of replication and expression. However, these genomes continue to function in an integrated manner, maintaining probably the closest and most important symbioses in the history of life (Sloan et al., 2018).

In a long-term evolutionary process, mitochondria and plastids were transformed from free-living bacteria to endosymbionts and organelles. Although the mitochondrial and plastid genomes were extremely reduced, they were not completely lost. This process raises the question of why evolution favored the reduction of cytoplasmic genomes and the transfer of genetic control to the nucleus, and why this process never stopped completely (Sloan et al., 2018). Importantly, many multisubunit enzyme complexes are formed by genes encoded by both cytoplasmic and nuclear genomes.

They are an important element of cyto-nuclear integration (Rand et al., 2004). Rapid evolution of the cytoplasmic genome can lead to changes in nuclear proteins and enzyme complexes (Van Der Sluis et al., 2015). Studies show that cyto-nuclear enzyme complexes are an arena for molecular co-evolution, and that nuclear-encoded subunits in these complexes evolve more rapidly than other nuclear-encoded proteins (Barreto & Burton, 2012; Grossman et al., 2004; Willett and Burton, 2004). Alternative explanations include that these subunits are not under strong functional constraints, or that selection for efficiency is less intense for mitochondrial than for nuclear processes (Pett & Lavrov, 2015; Sloan et al., 2014).

### 3.4.1 Cytonuclear interactions in polyploids

Polyploid lineages face the problem of coordinating the expression of genes involved in cytonuclear interactions after doubling the number of nuclear gene copies as the organelle genome is not immediately affected (Birchler &Veitia, 2012). However, the number of mitochondrial and plastid genomes is expected to increase in order to restore the balance in the stoichiometry of cytonuclear complexes (Sharbrough et al., 2017). Further mechanisms that maintain coordinated expression include decreased expression of organelle-targeted genes in the nucleus and increased organelle biogenesis. Polyploidy may also affect cytoplasmic interactions by reverting organelle-targeted nuclear genes to

a single copy after WGD (Sharbrough et al., 2017).

Furthermore, the well-documented positive correlation between nuclear genome size and cell size suggests that plants may be capable of accommodating a greater number of organelles within a single cell as nuclear genome copy number and cell size increase. indeed, plants with a greater number of genome copies exhibit an elevated number of chloroplasts per cell (Coate et al., 2011). Additionally, there are signalling pathways involved in organelle biogenesis and DNA replication that may play a role in coordinating cytosolic stoichiometry throughout genome duplication. Nevertheless, there is a paucity of systematic studies investigating the manner in which organelle number and size, cytoplasmic genome copy number, cytoplasmic gene expression levels, and signalling pathways alter after genome duplication (Sharbrough et al., 2017). Research using RNAseq has shown that the total amount of nuclear transcription directed to plastids decreases as ploidy increases, while the amount of transcription directed to mitochondria increases. Transcriptional coordination between nuclear-encoded genes and organelle genes is more conserved in plastids than in mitochondria as ploidy increases (Coate, et al., 2020).

### 3.4.2 Cytonuclear interactions in hybrids

Allopolyploidization is an important factor in the evolution of plant species. This process involves the combination of the genomes of distinct species and can lead to changes in the genome (Buggs et al., 2012), gene expression (Flagel & Wendel, 2010), and epigenetics (Paun et al., 2010). The interactions between the nuclear genome and organelle genomes are complex, with cytonuclear interactions being important in generating different phenotypes and increasing the competitiveness of organisms. Allopolyploid organism also has to resolve an issue related to the cytonuclear conflict. It is manifested by uniparental inheritance of the cytoplasmic genome, usually from the maternal parent, while nuclear genes originate from both parents. One factor affecting gene expression is the difference between alleles from the nuclear genome that target organelles or their promoter domains (Sharbrough et al., 2017).

Rubisco studies in different plant lineages show differential conservation of homologous genes, unbalanced expression, and asymmetric gene conversion in favor of maternal homologs (Gong et al., 2012). New research on cytonuclear adaptation in allopolyploid species both confirms and challenges previous assumptions about maternal

bias. Some allopolyploids show maternal expression bias for some cytonuclear genes, while others do not (Grover et al., 2022).

## 3.5 Cytonuclear complexes

In this project, we decided to study cytonuclear interactions of ATP synthase, Photosystem II, and RuBisCo. All of these complexes are important in the process of photosynthesis. Each of these protein complexes is built by proteins encoded by both nuclear and chloroplast genomes. For the purpose of this project, one nuclear-encoded gene and one chloroplast-encoded gene were selected for each complex.

### 3.5.1 ATP synthase

ATP synthase is a multi-subunit enzyme located in the cristae and inner membrane of mitochondria, chloroplast thylakoids, and bacterial plasma membranes (Walker, 2013). Except for the fact that light energy excites electrons to allow transmembrane movement of $H^+$ ions in chloroplasts, the structure and process of ATP synthesis is similar in all three locations (Hahn et al., 2018).

The ATP synthase consists of several subunits that form two main complexes, $CF_0$ and $CF_1$ (C as in chloroplast) (Kohzuma et al., 2017; Walker, 2013). An inner membrane-bound $F_0$ domain is involved in proton translocation, whereas an outer membrane-bound $F_1$ domain is a water-soluble catalytic domain. The $F_1$ domain consists of 8 subunits 3α, 3β, γ, δ a ε. The 3α and 3β form a hexametric ring around the γ, δ, and ε subunits which form the central rotor shaft. Subunits γ and ε bind together to the $F_0$. The $F_0$ domain is composed of four parts IV/a, I/b, II/b', and III/c (Figure 4) (Malik Ghulam et al., 2012; Neupane et al., 2019).

The $F_1$ domain subunits α, β, γ, δ a ε are encoded by genes *ATPA, ATPB, ATPC, ATPD* and *ATPE*. The $F_0$ contains 4 subunits IV/a, I/b, II/b', and III/c which are encoded by *ATPI, ATPF, ATPG,* and *ATPH* genes (Malik Ghulam et al., 2012).

In this thesis, I focus on the *ATPC1* and *ATPI* genes. *ATPC1* is a nuclear gene that encodes the $\gamma_1$ subunit of the $F_1$ part of the enzyme. *Arabidopsis thaliana* has two paralogs of this gene, *ATPC1* and *ATPC2*, which encode γ1 and γ2 respectively. The presence of both paralogs is not common throughout the plant kingdom, but it's not unique to *A. thaliana*. It has been proposed that these paralogs have different regulatory roles

(Kohzuma et al., 2012, 2017).

The *ATPI* gene is located in the chloroplast genome. In *A. thaliana*, the ATP genes are divided into two operons, Large (atp1) and Small (atp2). The Large operon encodes four genes *ATPI/H/F/A*, whereas the Small operon encodes only two genes *ATPB/E* (Malik Ghulam et al., 2012). Studies in Bacillus have suggested that ATPI is involved in translocating divalent cations and assisting in the internal transport of $Mg^{2+}$ (Hicks et al., 2003).

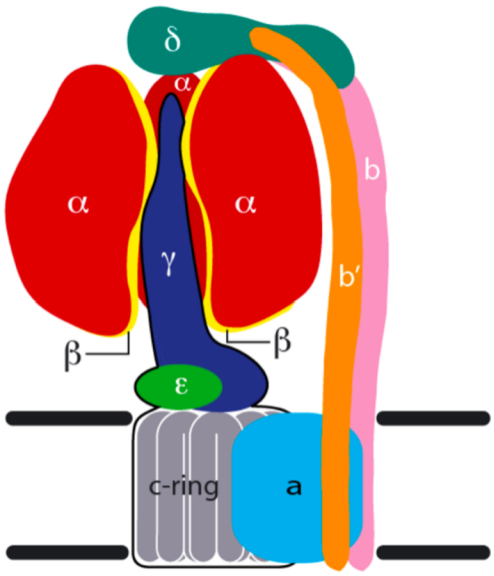



**Figure 4:** Schematics showing the arrangement of the protein subunits of the enzyme ATP synthase (Adopted from Neupane et al., 2019)

### 3.5.2 Photosystem II

Photosystem II (PSII) is a protein complex that plays a crucial role in the light reactions of photosynthesis. It is found in prokaryotic and eukaryotic organisms that can photosynthesize using oxygen, including higher plants, macroalgae, diatoms, dinoflagellates, and oxyphotobacteria (cyanobacteria and prochlorophyta), but not in archaea (Williamson et at., 2011).

Photosystem II (PSII) is located inside the thylakoid membrane and is responsible for light-driven water oxidation. This process releases an oxygen molecule and protons. PSII consists of several protein subunits, chlorophylls, quinone, and redox cofactors. The reaction center of PSII consists of proteins D1 and D2, which contain or bind redox

cofactors necessary for electron transfer (Figure 5) (Govindjee et al., 2010).

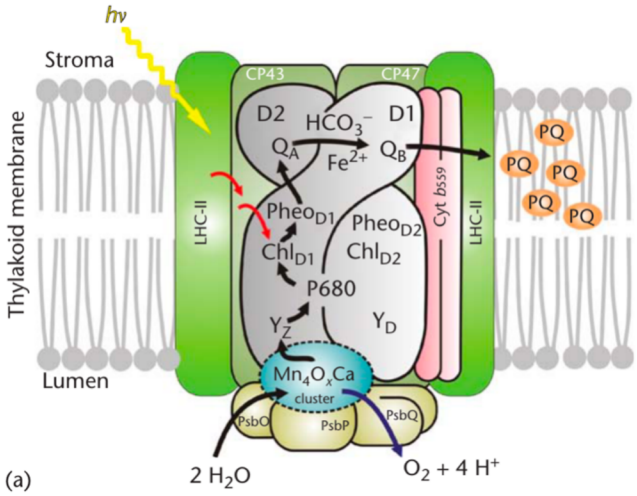



**Figure 5:** The schematic representation of Photosystem II in higher plants and algae (Adoptetd from Govindjee et al., 2010)

PSII consists of more than 20 proteins. In this thesis, I focus only on the *PSBQ1, PSBR,* and *PSBD* genes. The *PSBQ1* and *PSBR* are nuclear-encoded genes (Suorsa et al., 2006). *PSBQ1* is a member of the *PSBQ* family. In Arabidopsis, there are two *PSBQ* genes, *PSBQ1* and *PSBQ1*, and 3 *psbQ*-like homologs (*PQL*) (Ifuku, 2014). The products of the *PSBQ* gene family are involved in the process of oxygen evolution (Govindjee et al., 2010; H. Li et al., 2024). It has been proposed that the *PSBR* gene products are also involved in oxygen evolution. The oxygen evolution capacity of thylakoid membranes was shown to be reduced in the absence of *PSBR*. In conclusion, *PSBR* is essential for the optimization of photosynthetic water splitting and electron transfer in PSII (Suorsa et al., 2006).

The *PSBD* gene is located in the chloroplast genome and encodes the D2 protein of the PSII reaction center core complex. Together with the *PSBA* gene, which encodes the D1 protein, these genes are highly expressed, and their expression is thought to be light-dependent (Tsunoyama et al., 2004).

### 3.5.3 RuBisCo

Ribulose-1,5-bisphosphate carboxylase/oxygenase, or RuBisCo for short, is an enzyme involved in the light-independent (or "dark") part of photosynthesis, which facilitates carbon fixation by converting atmospheric carbon dioxide into high-energy molecules such as glucose.

RuBisCo consists of only two subunits, LS and SS, which are encoded by the

chloroplast *RBCL* gene, and a small family of two adjacent nuclear genes, *RBCS1* and *RBCS2* (Choquet & Wollman, 2023). It has a complex structure consisting of a smaller amino-terminal domain and a larger carboxy-terminal domain. The active site is located at the carboxy-terminal end of the b-chains and is complemented by residues from the amino-terminal domain of the adjacent large subunit. The overall structure of Rubisco is formed by the L2 dimer of the large subunits, which contain two active sites. The structure of the large subunits is largely well conserved, but there are minor differences between different forms of Rubisco.

Large subunits have a similar structure, whereas small subunits are more diverse. The basic structure consists of a four-stranded antiparallel β-chain covered by two helices. Significant differences are found in the loop between the A and B chains of the small subunits, known as the βA–βB loop, and at the carboxyterminus. The βA–βB loops of the four small subunits flank the holes of the solvent channel. Rubisco from prokaryotes and non-green algae has ten residues in the loop, whereas higher plants have 22 and green algae 28 (Andersson &Backlund, 2008).

### 3.5.4 Cytochrome B6f

The cytochrome B6f is one of the heterooligomeric proteins in photosynthetic membranes and is responsible for electron transport and energy transduction. It has an important role in the electronic connection between photosystem I and II (Baniulis et al., 2008). Cytochrome b6f is comprised of four major subunits: cytochrome b6, cytochrome f, the Rieske iron-sulfur protein, and subunit IV. This thesis examines the nuclear-encoded gene *PETC* and the plastid-encoded gene *PETA*. Both genes encode products that are involved in the cytochrome f subunit (Schneider et al., 2004).

# 4 Materials and methods

## 4.1 Materials and tools

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

### 4.1.1 Laboratory equipment

- 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 V ariomax (Electronicrührer)
- microwave oven (Zanussi)
- Nanodrop ND-1000 Spectrophotometer (Thermo Scientific)
- phytotron chamber (Wiss Gallenkamp)
- qPCR thermocycler (Bio-Rad)
- Qubit 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

- Geneious
- Imaris
- LaxX
- Maestro
- Microsoft Excel

- QuantaSoft
- Rstudio

### 4.1.3 Chemicals

The composition of buffers is summarized in Table 1.

**Table 1:** Composition of used buffers

| Buffer | Ingredient | Detergent (v/v) | Concentration |
|---|---|---|---|
| 1x TAE pH 8 | Tris | | 0,04 $mol \cdot l^{-1}$ |
| | EDTA | | 0,02 $mol \cdot l^{-1}$ |
| | H2O | | - |

- 10X DreamTaq Green Buffer (Thermo Scientific, cat. n. EP0714)
- 6X DNA Loading Dye (Thermo Fisher Scientific, cat. n. R0611)
- 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)
- 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)
- qPCR Supermix
- One Shot™ TOP10 chemically Competent cells E. coli (Invitrogena by Thermo Fisher Scientific, cat. n. C404010)
- paraformaldehyde (Sigma-Aldrich, cat. n. 30525-89-4)
- QX200 ddPCR EvaGreen Supermix (BioRad, cat. n. 1864034)
- sucrose (Lach-ner, cat. n. 10135-AP0)
- TAE buffer

#### 4.1.3.1 Chemical kits

- CloneJET PCR Cloning Kit (Thermo Fisher Scientific, cat. n. K1231)
- NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, cat. n. 740609.250)
- QIAprep® Spin Miniprep Kit (QIAGEN, cat. n.: 27104)
- RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, cat. n.: K1621)
- RNeasy kit (QIAGEN, cat. n.: 74104)

#### 4.1.3.2 Enzymes

- DreamTaq DNA Polymerase (Thermo Scientific, cat. n. EP071)
- HindIII restriction endonuclease (New England BioLabs, cat. n. R0104S)

#### 4.1.3.3 Oligonucleotides

Oligonucleotides used for ddPCR amplification are summarized in Tables 2-3 and oligonucleotides used for qPCR amplification are in Table 4.

**Table 2:** Oligonucleotides used for ddPCR (amplification of *Festuca pratensis* and *Lolium multiflorum* DNA fragments)

| Name | Sequence (5' ⟶ 3') |
|---|---|
| *ACTIN F* | CAAGCCGTCCTTTCCCTATATGC |
| *ACTIN* R | GAGTACCCTCTCTCAGTCAGGATC |
| *ATPC1* F | CAAGTTCGTCTCCCTCGTCCG |
| *ATPC1* R | TGCGTCTCGATCTTGATCTTCTCG |
| *ATPI* F | CGCTTAGCTTTCGACTTTTTGGG |
| *ATPI* R | ATGATGACCCTCCATGGATTCAC |
| *NDHB* R | GCGTTTCATTTGCTTCTCTTCAATG |
| *NDHB* R | GCTAATTATAGGTTCTTCTCTCCATC |
| *PSBD* F | CACTCTATTTGAGGACGGTGATGG |
| *PSBD* R | CTACGCCAATAGCACTCATCCAT |

| | |
|---|---|
| *PSBQ1* F | CTCGACGGGGTAAACATCTAACA |
| *PSBQ1* R | TCTTGATCTTGGCTGCATGGTC |
| *RBCL* F | GCGAAATGACTTTAGGTTTTGTTG |
| *RBCL* R | CCAAATTGTAATACAGAATCATCCC |

**Table 3:** Oligonucleotides used for ddPCR (amplification of *Arabidopsis* DNA fragments)

| Name | Sequence (5' ⟶ 3') |
|---|---|
| *ACTIN2* F | CCTGTTCTTCTTACCGAGGC |
| *ACTIN2* R | CAAGGTCAAGACGGAGGATG |
| *ATPC1* F | TGGTCAAATCAGAACCCGTG |
| *ATPC1* R | GAACAGGGTCTTGCTCGAAT |
| *ATPI* F | GGTTCCGCAGTTCTAACGAT |
| *ATPI* R | TTTCCACGGTAAAAGGGCTC |
| *NDHB* R | GCGTTTCATTTGCTTCTCTTCAATG |
| *NDHB* R | GCTAATTATAGGTTCTTCTCTCCATC |
| *PETA* F | GGTCCTGTTCCTGGTCAAAA |
| *PETA* R | TCCCCTGTTTCCACCTACAT |
| *PETC* F | AGTATATGGCTTCTTTGGTGTTACT |
| *PETC* R | TGAGTCTTAAGCCATTCCGC |
| *PSBD* F | TTCAATGGTCACCGCTAACC |
| *PSBD* R | TAGGCACGTAGGTTCAAAGC |
| *PSBR* F | CCCTCGTATTTGAAGCCACA |
| *PSBR* R | ATTTTGAAGGAGGGACGAGC |

**Table 4:** Oligonucleotides used for qPCR (amplification of *Arabidopsis* cDNA fragments)

| Name | Sequence (5' ⟶ 3') |
|---|---|
| *ACTIN* F | CCTGTTCTTCTTACCGAGGC |
| *ACTIN* R | AGAATCCAGCACAATACCGG |
| *ATPC1* F | TGGTCAAATCAGAACCCGTG |

| | |
|---|---|
| *ATPC1* R | GAACAGGGTCTTGCTCGAAT |
| *ATPI* F | GGTTCCGCAGTTCTAACGAT |
| *ATPI* R | TTTCCACGGTAAAAGGGCTC |
| *MATK* R | TAGGGTTGCTCAAGGATCCT |
| *MATK* R | GACCTTTTGCGATTGAAACCA |
| *PETA* F | GGTCCTGTTCCTGGTCAAAA |
| *PETA* R | TCCCCTGTTTCCACCTACAT |
| *PETC* F | AGAATTTCTTGTCAAGCGTCGAG |
| *PETC* R | TGAGTCTTAAGCCATTCCGC |
| *PSBD* F | TTCAATGGTCACCGCTAACC |
| *PSBD* R | TAGGCACGTAGGTTCAAAGC |
| *PSBR* F | CGACAAGCCCTTCGGAATTA |
| *PSBR* R | CCTTGTACACGTCACCACTC |

### 4.1.4 Plant material

Plants used in this project were grown in a controlled environment in a phytotron set to the following conditions 16h light, 8h dark, 20°C during the light phase and 16°C during the dark phase of the cycle, and 60% humidity. For the purposes of this thesis, two sets of plants were used, the *Festuca-Lolium* group (Table 5 –7) and the *Arabidopsis* group consisting of *A. thaliana* and *A. lyrata*. The *Arabidopsis* group consisted of 48 plants, of which 12 were diploid progenitors, 24 were autopolyploids and 12 were allopolyploids.

**Table 5:** Festuca pratensis (1st and 7th generation after colchicine treatment (C1 and C7)

| Ploidy | Cultivar | Ploidy | Cultivar (C1 plants) |
|---|---|---|---|
| | Hyperbola | | Hyperbola |
| | Praniza | | Praniza |
| 2x | 48616 | 4x | 48616 |
| | 24189 | | 24189 |
| | Kolumbus | | Kolumbus |

| Ploidy | Cultivar | Ploidy | Cultivar (C7 plants) |
|---|---|---|---|
| | Fure | | S2 Moestr |
| | Tomosake | | Raskilla |
| 2x | Cosmonaut | 4x | Patra |
| | Kolumbus | | Tetrax |
| | Preval | | Westa |

continuation of the Table 5

**Table 6:** Lolium multiflorum (7th generation after colchicine treatment)

| Ploidy | Cultivar | Ploidy | Cultivar (C7 plants) |
|---|---|---|---|
| 2x | Partax | 4x | Pepper |
| | Tiger | | Dallara |
| | Barherta | | Passat |
| | Yolande | | Firkin |
| | Skippy | | Mitos |

**Table 7:** Festuca x Lolium hybrids (F1 hybrids)

| 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 | | | | |
| Parent plants | Lm MATIZ 74/11 | | | | |
| | Fp WSC 62/11 | | | | |

## 4.2 Experimental procedures

### 4.2.1 Cell morphology analysis

#### 4.2.1.1 Microscopy slide preparation

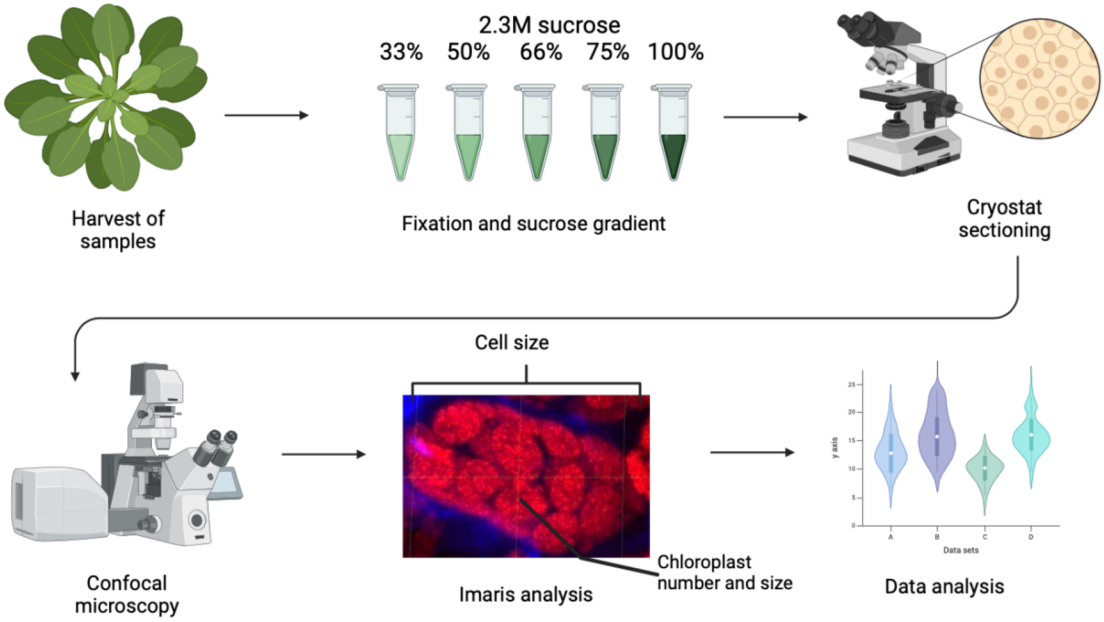



**Figure 6:** Cell morphology analysis workflow

##### 4.2.1.1.1 Plant tissue fixation and dehydration

The second layer of leaves was excised from one-month-old plants and fixed for 2h at room temperature in a solution containing 4 % paraformaldehyde and 1x phosphate-buffered saline (PBS; pH 7.0). The samples were then dehydrated in a sucrose gradient. The dehydration steps with 25 %, 33 %, 50 %, 66 %, and 75 % 2.3 M sucrose were performed for 1h each at room temperature. Finally, the samples were left in 100 % 2.3 M sucrose overnight at 4°C.

#### 4.2.1.2 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 labeled with fluorescent dye DAPI. The slides were stored in the dark at 4°C until imagining.

#### 4.2.1.3 Image evaluation in Imaris software

Images were visualized in Imaris software. The number of chloroplasts, the size and volume of the cells, the size and volume of chloroplasts, and the volume of the nucleus were examined. Generally, six plants were analyzed for each ploidy and 25 cells from each plant were examined for the number of chloroplasts and the cell and chloroplast volume. the number of nuclei examined in each species varied based on their visibility in the microscope. The Autopolyploid set contained 12 diploid and 18 polyploid plants (12 neopolyploids and 6 established polyploids). These were clones of the single plant treated with colchicine, where one plant was diploid, and one plant was tetraploid. The allopolyploid set contained 10 plants. Data analysis was carried out using R studio.

#### 4.2.1.4 Data analysis

All the data obtained were entered into Excel (Abbrevation 1), where means and standard errors were calculated for the observed characteristics. The graphs and statistical analysis were generated in R Studio. The Cohen D statistical test was used for this analysis. Cohen's d is a standardized effect size used to measure the difference between two group means. Cohen's d is calculated by taking the difference between the two means and dividing it by the standard deviation of the data. This measure reports the size of the difference between the means in comparison to the variability of the data (negligible effect $d < 0.2$; small effect $d = 0.2–0.5$; moderate effect $d = 0.5–0.8$; large effect $d \geq 0.8$).

### 4.2.2 Gene copy number determination in auto- and allopolyploids

#### 4.2.2.1 Gene selection

Initially, the chloroplast-encoded genes and their nuclear-encoded counterparts were selected from four cytonuclear complexes (Table 8). Non-interacting genes were used as controls/references). Oligonucleotides were designed by my supervisor (Tables 2–4).

**Table 8:** A list of selected genes

| Species | Plastid | Nuclear | Complex | Non-interacting |
|---|---|---|---|---|
| *L. multiflorum*<br>*F. pratensis* | *ATPI* | *ATPC1* | ATP synthase | *actin* (nucleus) |
| | *PSBD* | *PSBQ1* | Photosystem II | *ndhB* (plastid) |

| | *RBCL* | *RBCS* | RuBisCo | |
|---|---|---|---|---|
| **Species** | **Plastid** | **Nuclear** | **Complex** | **Non-interacting** |
| *A. thaliana.* *A. lyrata* | *ATPI* | *ATPC1* | ATP synthase | *actin* (nucleus) |
| | *PSBD* | *PSBR* | Photosystem II | *ndhB* (plastid – ddPCR) |
| | *PETA* | *PETC* | Cytochrom B6f | *matK* (plastid – qPCR) |

#### 4.2.2.2 ddPCR

To estimate the change in chloroplast genome copy number after WGD, droplet digital PCR (ddPCR) was used. The DNA was isolated using the NucleoSpin Plant II kit following the manufacturer's instructions. The optimal conditions for ddPCR were determined during previous work on my bachelor's thesis. The genomic DNA was digested with the restriction enzyme HindIII (Table 9). The optimal annealing temperature for oligonucleotides and suitable working concentrations of DNA was determined using the gradient ddPCR. Optimal working concentrations of *Festuca-Lolium* DNA were 10 ng·µl$^{-1}$ for nuclear genes and 0,1 ng·µl$^{-1}$ for chloroplast genes. Optimal concentrations of *Arabidopsis* DNA were 5 ng·µl$^{-1}$ for nuclear genes and 0,01 ng·µl$^{-1}$ for chloroplast genes. The optimal annealing temperature for *Festuca-Lolium* primers was 58°C and for *Arabidopsis* primers 59°C.

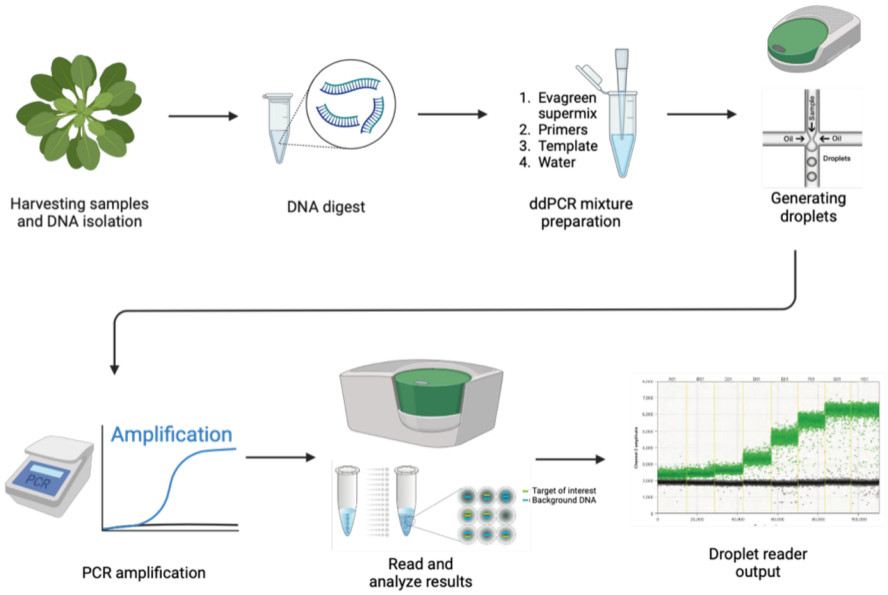



**Figure 7:** ddPCR workflow

**Table 9:** 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** |

**Table 10:** ddPCR reaction mix

| Stock solution | Stock solution concentration | Work concentration | Volume [µl] |
|---|---|---|---|
| EvaGreen Supermix | 2x | 1x | 11 |
| Forward primer | 50µM | 10µM | 1 |
| Reverse primer | 50µM | 10µM | 1 |
| gDNA | sample dependent | 0,1/ 10 ng·µl$^{-1}$ | 1 |
| $H_2O$ | - | - | 9.46 |
| **total volume** | | | **22** |

ddPCR reactions were prepared (Table 10) and the droplets were generated according to the Manufacturer's instructions. In brief: A droplet-generating cartridge was inserted and snapped into the stand (DG8TM QX100TM / QX200TM Drop Generator Cartridges). 60 µl of droplet generator oil was pipetted into the lower wells (labeled "Oil"). 20 µl of the reaction mixture were gently pipetted into the middle wells. After checking and removing any bubbles, the rubber band was pulled over and the cassette was inserted into the droplet generator. After the droplet formation, 40 µl of the emulsion mixture (reaction mixture and oil) was aspirated from the upper wells of the cartridge very gently 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 sealed using pierceable aluminum sealing foil, placed in a deep well thermocycler, and the program for ddPCR was started (Table 11).

**Table 11:** ddPCR cycling conditions

| Steps | Cycles | Temperatures | Time |
|---|---|---|---|
| Initial denaturation | 1 | 95°C | 10 minutes |
| Denaturation | 40 | 95°C | 30 seconds |
| Aneling | | Sample dependent | 30 seconds |
| Extension | | 98°C | 1 minute |

After completion of the PCR program, the plate was inserted into the droplet analyzer 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 positive droplets and the gene copy number in the sample was analytically calculated.

#### 4.2.2.3 Data analysis

The data set provided by the Droplet Reader was exported in .csv format and analyzed using R Studio. The copy number of nuclear genes per sample was divided by the sample ploidy to estimate the number of nuclei in the reaction. The copy number of chloroplast genes in the sample was divided by the number of nuclei to estimate the number of chloroplast genes per cell. The results of *NDHB* were divided by two to take into account that it is a two-copy gene. The experiment consisted of four biological and two technical replicates and was repeated twice.

### 4.2.3 Gene expression analysis

#### 4.2.3.1 qPCR

Total mRNA samples were harvested from the second leaf layer and isolated using RNeasy Plant Mini kit. The quantity and quality of the RNA samples were measured using Qubit Fluorometrer, Nanodrop ND-1000 spectrophotometer and 1% agarose gel

**Figure 8:** qPCR workflow

electrophoresis. Samples were then treated with DNase and cDNA was generated using RevertAid First Strand cDNA Synthesis Kit. cDNA quality was checked by Nanodrop. The primers were designed with a melting temperature (Tm) of 58°C. *ACTIN* was selected as a nuclear reference gene and *MATK* as a chloroplast *reference gene*. The qPCR reaction mixture was prepared according to Table 16. The reaction was then transferred into qPCR thermocycler and the program started (Table 17).

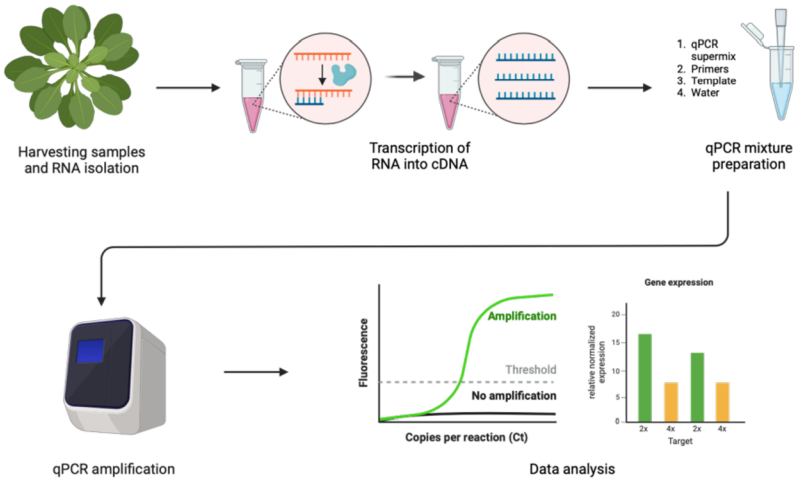



**Table 12:** qPCR reaction mix

| Stock solution | Stock solution concentration | Work concentration | Volume [µl] |
|---|---|---|---|
| Master mix (syber green) | 1x | 1x | 7.5 |
| Forward primer | 50µM | 5 pM | 0.5 |
| Reverse primer | 50µM | 5 pM | 0.5 |
| cDNA | sample dependent | 10 ng·µl$^{-1}$ | 1 |
| $H_2O$ | - | - | 5.5 |
| **total volume** | | | **15** |

**Table 13:** qPCR cycling program

| Steps | Cycles | Temperatures | Time |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Initial denaturation | 1 | 94°C | 5 minutes |
| Denaturation | 35 | 94°C | 10 seconds |
| Aneling | | 58°C | 10 seconds |
| Extension | | 72°C | 30 seconds |
| Melt curve | 1 | 65-95°C | |

#### 4.2.3.2 Data analysis

The qPCR experiment involves several steps, from setting up the protocol, preparing the plate, and analyzing the data. Samples for gene expression analysis consist of unknown samples (target genes), positive controls (reference genes), and non-template controls (NTC). After the qPCR run is completed, the initial data analysis is conducted using CFX Maestro, complemented by R Studio for data visualization. Within the software, the data analysis window includes tabs for quantification data, melting curves, gene expression data, and run information to evaluate the quality and quantity of the amplification process. Assessment of the PCR products specificity should be done by melt curve analysis followed by Sanger sequencing. To ensure product size and purity, it is standard practice to run the PCR products on an agarose gel after the qPCR reaction. After confirming the specificity of the amplicons, we need to measure the efficiency of the primers for accurate Cq value determination and data analysis. Primer efficiency is measured by generating a standard curve through a series of dilutions, usually at a 1/10 dilution factor. After primer efficiency has been calculated, the next step is to determine the quantification cycle (Cq) values. The software provides a comprehensive data analysis framework that allows for the calculation of Cq values using either Regression or Single Threshold mode. In Regression mode, the software applies a multivariable, nonlinear regression model to individual well traces and then uses this model to compute an optimal Cq value. The $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001) was employed to analyze the transcript fold-changes relative to the reference using Maestro software v 2.0 (Bio–Rad, USA). After determining the accurate Cq values, the software calculates the P-value, which is a measure of the statistical significance between the differential expression of two biological groups. The P-value is calculated using an unpaired t-test that assumes equal variances between the groups under analysis. For data visualization, exporting the data to csv. format for further analysis in R Studio was performed using the ggplot2

library to create the bar charts.

# 5 Results

## 5.1 Cell morphology analysis

In the submitted thesis, we are looking for the intracellular changes associated with polyploidization, such as an increase in cell volume. The other thing we observed were the changes associated with the disruption of cytonuclear interactions, for example the increase in the number of chloroplasts, their volume or the number of chloroplast genomes. In total, I evaluated of *Arabidopsis* group (*A. thaliana* and *A. lyrata*) 42 plants of which 12 were diploid progenitors, 18 were autopolyploids, which included 12 nepoplyploid of A. thaliana and *A. lyrata,* and established polyploid of *A. thaliana*, and 12 were allopolyploids. Generally, 150 cells per ploidy were examined for chloroplast number, cell size, nuclear volume, and chloroplast size.

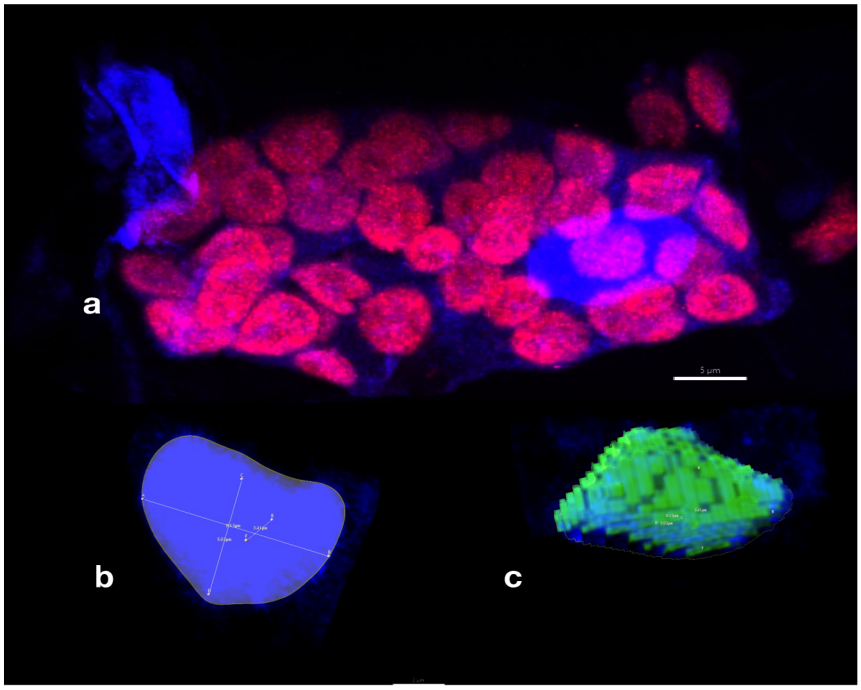



**Figure 9:** Visualization of a) tetraploid cell with clearly visible and recognizable chloroplasts, b) measurement of nucleus diameter, c) measurement of nucleus volume using surface function in Imaris software.

Cell morphology analysis was performed for *the Arabidopsis* complex in this thesis, *Festuca-Lolium* complex was analyzed previously as part of my bachelors project and published in (Shahbazi et al., 2024) The nucleus volume was determined using the "Surface" function based on the DAPI staining intensity and weren't visible in all evaluated cells, therefore the number of measured nuclei varies in each data set.

A difference in ploidy was observed for all parameters analyzed. In *A. lyrata*, the

cell volume of tetraploids was found to be 15993 ± 686 $\mu m^3$ (mean ± SE), whereas the diploid cell size was 17603 ± 743 $\mu m^3$. This indicates that tetraploids are on average 9.15 % smaller than diploids. The Cohen d test revealed that the magnitude of the difference was negligible in the case of *A. lyrata*, whereas in *A. thaliana*, tetraploids were approximately 92 % larger than diploids (diploids: 8630 ± 415 $\mu m^3$, tetraploids: 16612 ± 774 $\mu m^3$). The Cohen D test indicated a significant difference. The difference between the two groups was 40.62 %, with diploid cell size being 140258 ± 581 $\mu m^3$ and tetraploid cell size being 20126 ± 926 $\mu m^3$. The statistic Cohen D test is moderate. Furthermore, a distinction was observed between diploids and established polyploids (14097 ± 554 $\mu m^3$), with established polyploids exhibiting a 48.98% reduction in size (Cohen d – large) (Figure 10a).

In general, the nuclear volume was found to be lower in diploids than in tetraploids. The observed difference was 38.72 % in *A. lyrata* (diploids 204 ± 9 $\mu m^3$, tetraploids 283 ± 16 $\mu m^3$, Cohen's d moderate), 70.38 % in *A. thaliana* (diploids 260 ± 9 $\mu m^3$, tetraploids 443 ± 16 $\mu m^3$, Cohen's d large) and 37.95 % in hybrids (diploids 224 ± 9 $\mu m^3$, tetraploids 309 ± 14 $\mu m^3$, Cohen's d moderate). Nevertheless, the average nucleus volume of the established tetraploids was 226 ± 8 $\mu m^3$, representing a 48.98 % reduction in size compared to that of the diploids (Cohen's d small) (Figure 10b).

The number of chloroplasts was found to be higher in tetraploids in all groups analyzed. *A. lyrata* diploids were observed to contain on average 41 ± 0.77 (mean ± SE) chloroplasts per cell, whereas tetraploids were found to contain 54 ± 0.90 chloroplasts per cell. This represents a difference of 31.71 %. A greater difference was observed between *A. thaliana* diploids and tetraploids (46.67%). The number of chloroplasts was 30 ± 0.49 in diploids and 44 ± 0.88 in tetraploids. The mean number of chloroplasts per cell in diploid hybrids was 53 ± 0.86, while that of tetraploids was 57 ± 0.999. This represents a difference of 7.55 %. The greatest difference was observed between diploids (30 ± 0.49) and established tetraploids (60 ± 0.80) (Figure 10c). Cohen's d-test values were found to be statistically significant for all groups analyzed, except for the hybrids, where the test yielded a non-significant result.

There was no discernible pattern in the results for chloroplast volume. In *A. lyrata*, diploid chloroplast volume was 108 ± 4 $\mu m^3$ and tetraploid was 93 ± 2 $\mu m^3$, indicating that tetraploid chloroplasts were on average 13.89 % smaller. A similar reduction in volume of 12.79 % was observed in *A. thaliana*, with the volume of the chloroplasts being 86 ± 2 $\mu m^3$ in the diploids and 75 ± 3 $\mu m^3$ in the tetraploids. The chloroplast volume in

diploid hybrids was 92 ± 2 µm3 and in tetraploid hybrids 95 ± 2 µm$^3$. The observed difference between the two groups was 3.26 %. A similar chloroplast volume was observed between the diploids and the established polyploids (74 ± 2 µm$^3$), which was 1.35 % (Figure 10d). The results of the Cohen d statistical test were small for all the analysed groups, with the exception of the hybrids, which were negligible.

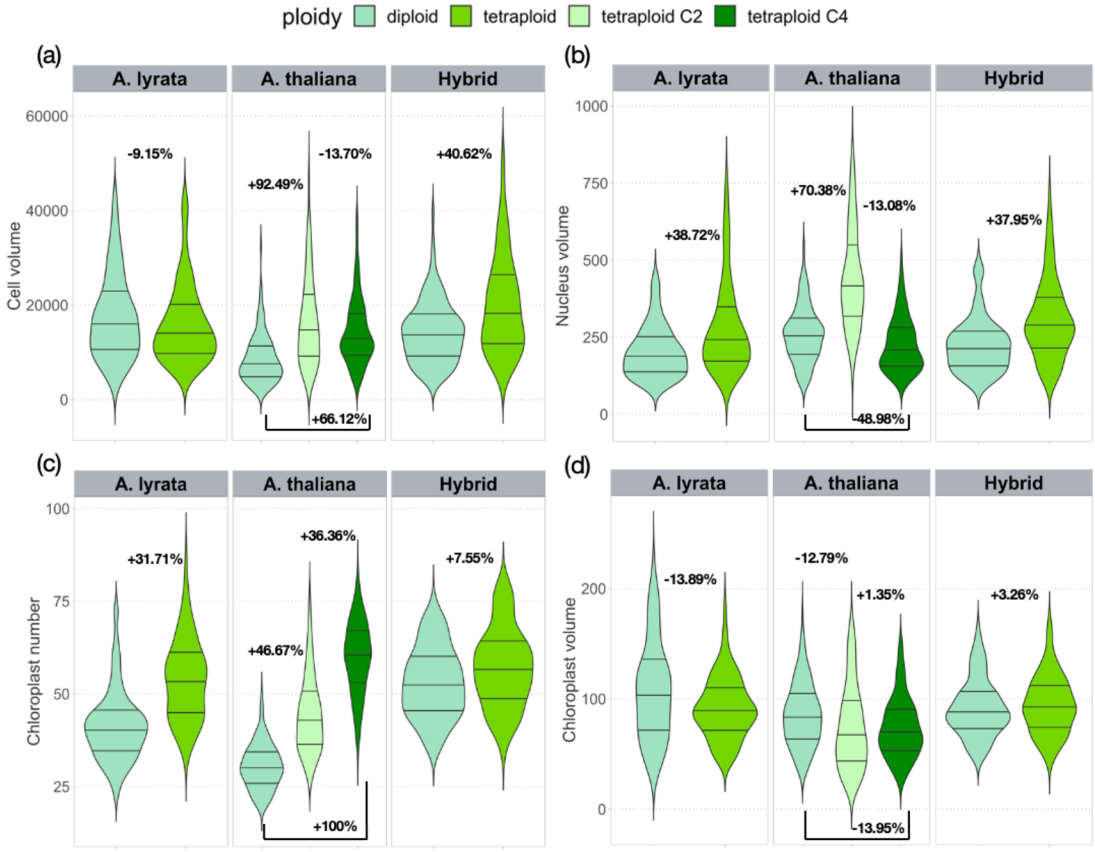



**Figure 10:** To visualize the results we used the violin plots. The middle line represents the median while the other two represent the lower and upper quartiles. The width of the graph indicates the number of individuals of a given volume or number. In this graph a visualized the changes in cell morphology after WGD in the *Arabidopsis* group. The y-axis represents (a) cell volume, (b) nucleus volume, (c) chloroplast number and (d) chloroplast volume. The difference between the two means is expressed as +% for an increase and -% for a decrease.

## 5.2 Determination of chloroplast genome copy number

The experiment was conducted on two sets of plants. The first set, the *Festuca-Lolium* group, consisted of two species: *Festuca pratensis* and *Lolium multiflorum*, as well as their hybrids. Autotetraploids were produced by colchicine treatment from diploid cultivars. The second set, the *Arabidopsis* group, consisted of *A. thaliana* and *A. lyrata*, and their hybrids. Autotetraploids were produced the same way as for the previous group of plants.

### 5.2.1 Festuca-Lolium group

The *Festuca-Lolium* group has been the first subject of analysis. To determine how the number of chloroplast genome copies changed after the WGD, were selected three chloroplast-encoded cytonuclear interacting genes *ATP synthase subunit I (ATPI), Photosystem II D2 protein chain (RBCL),* and *Ribulose bisphosphate carboxylase large chain (RBCL).* The copy number of these genes per cell for each ploidy was estimated using the droplet digital PCR (ddPCR) and the results were compared. To determine the number of cells in each sample we analyzed the number of copies per sample of the single-copy nuclear-encoded gene *actin 2 (ACT2).*

**Table 14:** The chloroplast genome copy number in *Festuca pratensis* and *Lolium multiflorum;* NE = newly established, SE = standard error of the mean

| | diploid mean | SE | tetraploid mean | SE |
|---|---|---|---|---|
| *Festuca pratensis* NE | 122 | 11 | 258 | 17 |
| *Festuca pratensis* | 583 | 56 | 1543 | 126 |
| *Lolium multiflorum* | 485 | 53 | 1202 | 95 |

The results (Table 14) showed that there was an increase in gene copy number between ploidies. Newly established *F. pratensis* tetraploids contained 111.48 % more copies of the chloroplast genome per cell than corresponding diploids (Figure 11a). Established tetraploid *F. pratensis* plants contained 163.67 % more copies per cell than their diploid counterparts (Figure 11b). *Lolium multiflorum* polyploids had 147.84 % more copies per cell than their diploid counterparts (Figure 11c). Overall, there was a 2.5-fold increase in established F. pratensis and *L. multiflorum* tetraploids compared to the diploids (P value <0.05; effect size based on Cohen's D test = large in all pairwise comparisons per gene), and a 2.1-fold increase in newly established *F. pratensis* tetraploids compared to the diploids (P value <0.05; effect size based on Cohen's D test = large in all pairwise comparisons per gene).

The data indicate that the imbalance in cytonuclear complexes after WGD is fully restored or even exceeded by the increase in chloroplast genome copies. This is achieved both by increasing the number of chloroplast genomes and by increasing the number of chloroplasts. An immediate increase in the copy number of chloroplast genomes after WGD is observed, which continues in subsequent generations.

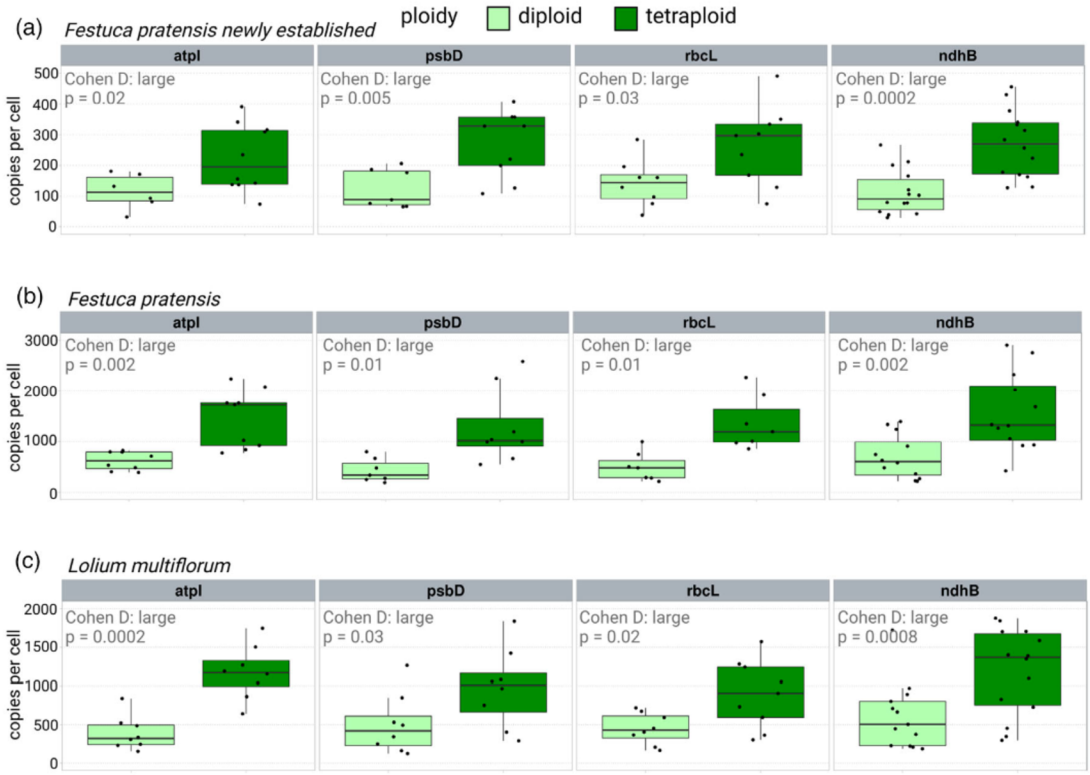



**Figure 11:** depicts the number of chloroplast genome copies in diploid plants (light green) and their autotetraploid counterparts (darker green) as determined by ddPCR. The plot represents the results of a number of chloroplast-encoded genes in newly established autotetraploid *F. pratensis* (a), well-established autotetraploid *F. pratensis* (b), and well-established autotetraploid *L. multiflorum* (c) plants. It should be noted that the y-axis represents the number of gene copies per cell. The chloroplast gene NAD(P)H-quinone oxidoreductase subunit 2 (NADH) was included as a control. Its values have been divided by two, as this gene has two copies per chloroplast genome.

### 5.2.2 Arabidopsis group

I started to optimize the analysis of chloroplast genome copy number changes in the Arabidopsis group, but there was not enough time to complete this part.

## 5.3 Determination of gene expression

qPCR was used to evaluate changes in gene expression after WGD of both chloroplast and nuclear-encoded genes involved in cytonuclear interactions. Analysis of the *Festuca-Lolium* group was reported in Shahbazi et al. 2024, therefore only the *Arabidopsis* group was assessed as part of this diploma thesis. The same set of genes

analyzed was used to determine changes in the number of chloroplast genomes.

To normalize the qPCR data at each ploidy level, we used the constitutively expressed *maturase K* (*MATK*) gene as the chloroplast reference and *ACT2* as the nuclear transcript reference to account for the increased expression caused by the higher number of chloroplast genomes in polyploids compared to diploids. For the purposes of this thesis, we use the method of relative quantification, which analyses changes in gene expression in a given sample relative to another reference sample (e.g. an untreated control sample). The reference sample we used was the diploid ancestor of the analyzed individual.

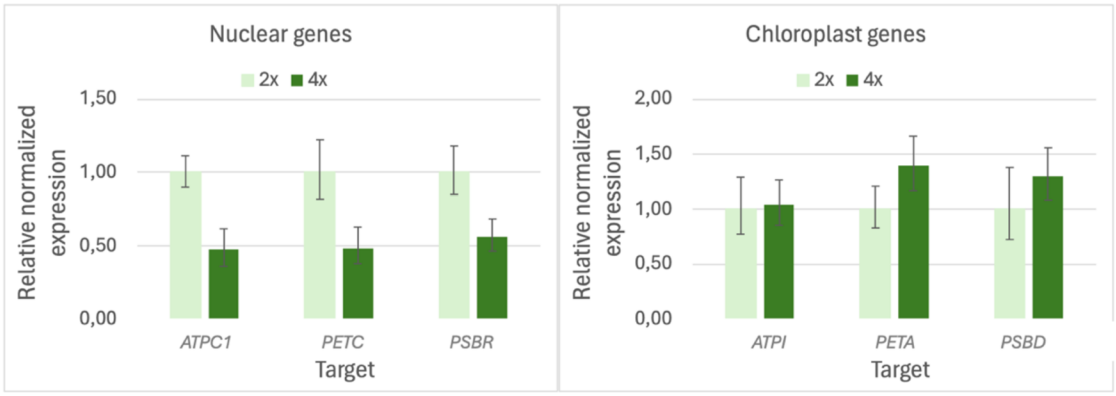



**Figure 12:** Plot showing changes in relative normalized expression between diploids and tetraploids of nuclear *ATPC1* (ATP synthase gamma chain 1), *PETC* (cytochrome b6), and *PSBR* (Photosystem II) and chloroplast *ATPI*, *PETA*, and *PSBD* genes involved in cytonuclear complexes in *A. thaliana*. Error bars represent the standard error of the mean of the biological replicates (n = 5).

In the pure species *A. thaliana* and *A. lyrata*, no significant up- or down-regulation was detected. The change in expression in *A. thalina* was not significant for any of the genes examined, neither nuclear nor chloroplast encoded. There was an average 50 % (*ATPC1* 53 %, *PETC* 51 %, *PSBR* 44 %) decrease in the expression of nuclear-encoded genes in tetraploids compared to diploids. The value of the relative normalized expression of *ATPC1* was 0.47 ± 0.61/0.36 (relative normalized expression/ upper and lower errors), there was the greatest difference in expression in this gene. The value of *PETC* was 0.48 ± 0.62/0.37 and that of *PSBR* was 0.56 ± 0.69/0.46. The difference in expression of chloroplast-encoded genes was not significant for any of the genes analyzed. There was a similar difference in the genes *PETA* (39 %) and *PSBD* (30 %). The expression of *ATPI* was almost the same in diploids and tetraploids (4 % difference). The relative normalized expression values of *PETA* and *PSBD* were 1.39 ± 1.66/1.17 and 1.29 ± 1.55/1.09, respectively. The expression value for *ATPI* was 1.04 ± 1.27/0.86. The expression of chloroplast genes was significantly up-regulated in the tetraploids compared to the

diploids. Specifically, *ATPI* showed a 177 % increase and *PETA* showed a 50% increase. The relative normalized expression value for *ATPI* was 2.77 ± 3.02/2.55 and for *PETA* was 1.50 ± 1.65/1.37. The expression of the *PSBD* gene showed a 71 % difference with a value of 1.71 ± 1.89/1.54 (Figure 12).

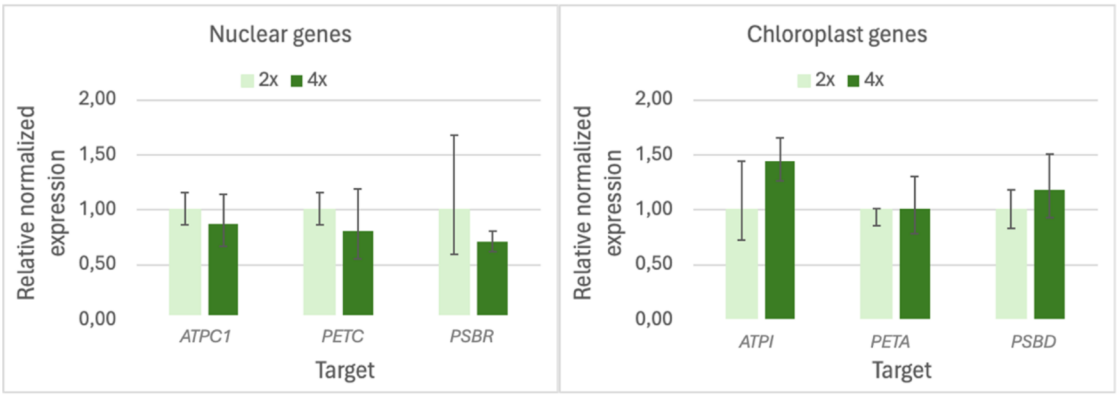



**Figure 13:** Plot showing changes in relative normalized expression between diploids and tetraploids of nuclear *ATPC1* (ATP synthase gamma chain 1), *PETC* (cytochrome b6), and *PSBR* (Photosystem II) and chloroplast *ATPI*, *PETA*, and *PSBD* genes involved in cytonuclear complexes in *A. lyrata*. Error bars represent the standard error of the mean of the biological replicates (n = 5).

In *A. lyrata*, the decrase in expression of nuclear-encoded genes was lower than in *A. thaliana*. The lower difference in the expression of *ATPC1* was -13 %, and the expression value of *PETC* was slightly higher (-19 %). The gene expression of *PSBR* was -29 %. The value of the relative normalized expression of *ATPC1* was 0.87 ± 1.16/0.86, there was the lowest difference in expression in this gene. The value of PETC was 0.81 ± 1.18/0.55 and that of PSBR was 0.71 ± 0.81/0.62. There was no significant difference in the expression of chloroplast-encoded genes, except for *ATPI* which showed a +44 % difference. The relative normalized expression of the other genes was lower than that of *ATPI*. The expression of *PSBD* changed by +18%, while that of *PETA* changed by only +1%. The values of the relative normalized expression of these genes were: *ATPI* 1.44 ± 1.65/1.25, *PSBD* 1.18 ± 1.51/0.93, and *PETA* 1.01 ± 1.31/0.79 (Figure 13).

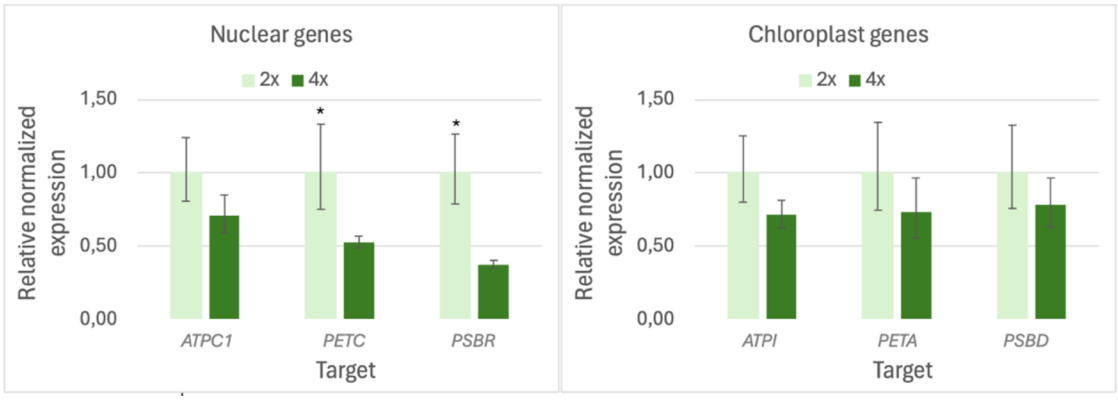



**Figure 14:** Plot showing changes in relative normalized expression between diploids and tetraploids of nuclear *ATPC1* (ATP synthase gamma chain 1), *PETC* (cytochrome b6), and *PSBR* (Photosystem II) and chloroplast *ATPI*, *PETA*, and *PSBD* genes involved in cytonuclear complexes in established polyploids *of A. thaliana*. Error bars represent the standard error of the mean of the biological replicates (n = 5). The stars above the bar indicate that the p-value was under 0.05.

The nuclear genes were significantly down-regulated in the established tetraploid. Gene *ATPC1* showed a non-significant increase of + 29 % with a value of 0.71 ± 0.85/0.59. Genes *PETC* and *PSBR* showed an increase in relative normalized expression of 47 % and 62 %, respectively. The value for *PETC* was 0.53 ± 0.57/0.49 and for *PSBR* was 0.38 ± 0.40/0.35. The expression of chloroplast genes did not show any significant decrease for the studied genes, with an average difference of around 30 % (*ATPI* 29 %, *PETA* 27 %, and *PSBD* 22 %). The relative normalized expression values were 0.71 ± 0.81/0.63 for *ATPI*, 0.73 ± 0.97/0.56 for *PETA*, and 0.78 ± 0.97/0.63 for *PSBD* (Figure 14).

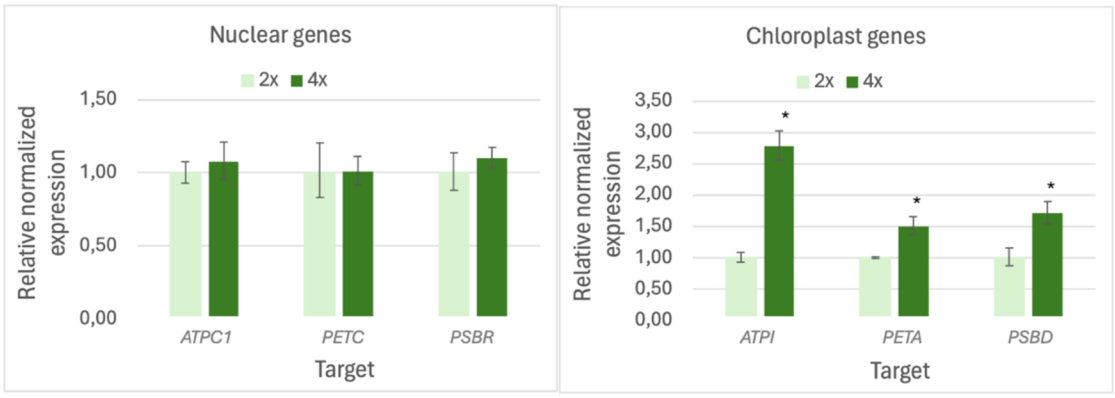



**Figure 15:** Plot showing changes in relative normalized expression between diploids and tetraploids of nuclear *ATPC1* (ATP synthase gamma chain 1), *PETC* (cytochrome b6), and *PSBR* (Photosystem II) and chloroplast *ATPI*, *PETA*, and *PSBD* genes involved in cytonuclear complexes in hybrids. Error bars represent the standard error of the mean of the biological replicates (n = 5). The stars above the bar indicate that the p-value was under 0.05.

In the hybrids, the expression of nuclear genes showed almost no variation. The expression of *ATPC1* showed an increase of + 8 %, with an expression value of 1.08 ± 1.21/0.96, while the expression of *PETC* showed a difference of only + 1 %, with an expression value of 1.10 ± 1.18/1.03. In the hybrids, the expression of nuclear genes showed almost no variation. The expression of *ATPC1* showed a difference of 8%, with an expression value of 1.08 ±1.21/0.96, while the expression of *PETC* showed a difference of only 1%, with an expression value of 1.10 ± 1.18/1.03. The expression of chloroplast genes was significantly up-regulated in the tetraploids compared to the diploids. Specifically, *ATPI* showed a 177% increase and *PETA* showed a 50% increase. The relative normalized expression value for *ATPI* was 2.77 ± 3.02/2.55 and for *PETA* was 1.50 ± 1.65/1.37. The expression of *PSBD* gene showed a 71% difference with a value of 1.71 ± 1.89/1.54 (Figure 15).

# 6 Discussion

The duplication of genetic information in the nucleus, also known as WGD, can cause massive changes in the genome and also in the nuclear architecture. Cell volume is the first characteristic that is generally expected to change after WGD. The difference in cell volume was observed in *A. thaliana*, where there was a 92.49 % increase in cell volume. This is well documented in many plant species (Bomblies, 2020). However, our measurements suggest that the difference decreases in subsequent generations. For established tetraploids, the increase was only 66.12 % compared to the diploids. This doesn't correlate with the results of Shahbazi et al., 2024 where the difference increases with subsequent generations. The rapid increase in cell volume in neopolyploids and the subsequent decrease in following generations of polyploids may be a sign of an attempt of an organism to return to the original state. In contrast, a 9.15 % decrease in cell volume was observed in *A. lyrata*. According to Tsukaya (2013), the relationship between ploidy and increase in cell volume is neither linear nor continuous. Various species can have different responses to the WGD, and plant can increase their cell volume without the change of ploidy (Kawade et al., 2013). This implies that polyploidy is only one of the factors which play a role in cell volume increase. Nuclear volume increased in all neopolyploids (70.38 % in *A. thaliana*, 38.72% in *A. lyrata,* and 37.95 % in hybrids). However, the increase in nuclear volume was notably lower than the increase in cell volume. Again, as with cell volume, a decrease in nucleus volume can be observed in subsequent generations.

WGD has significant implications for the balance of cytonuclear interactions. A number of crucial complexes are composed of products from both nuclear-encoded and organellar-encoded genes. For example, RuBisCo is composed of a large subunit encoded by an organellar gene, *rbcL,* and a small subunit encoded by a nuclear gene *rbcS* (Andersson & Backlund, 2008). Polyploidization results in the duplication of nuclear information, while the information in the organelles remains unaltered. The polyploid organism can adapt to this imbalance by increasing the number of organelles (Doyle & Coate, 2019; Shahbazi et al., 2024). This study clearly shows that there is an increase in the number of chloroplasts in all groups studied, both neopolyploids and established polyploids. Notably, the increase in *A. thaliana* neopolyploids was 1.5-fold which further increased to 2-fold between diploids and established tetraploids. The increase between neopolyploids and established polyploids was also observed in the *Festuca-Lolim* group,

but the difference wasn't as pronounced as in the Arabidopsis group (from a 15% increase to 33.5%). The increase in neopolyploids observed in this study is lower than that reported in other species. For instance, in *Triticum* (Pyke & Leech, 1987) or sugar beet, the number of chloroplasts increased by up to 72% (Mochizuki et al., 1955). However, this study suggests that increasing the number of chloroplasts is one but not the only strategy that plants can use to overcome the imbalance in cytonuclear interactions after polyploidization.

Another such strategy is to change the number of organellar genomes (Shahbazi et al., 2024; Sharbrough et al., 2017). Increasing the number of organellar genomes can potentially increase the size of the organelle itself. However, here we observed that the change in chloroplast volume was negligible and irregular. In pure species, there was a decrease in chloroplast volume of about 13% in the tetraploids compared to the diploids. On the other hand, there was an increase in chloroplast volume in subsequent generations and hybrids. This may indicate that a change in this characteristic is not essential in the process of addressing the imbalance following the WGD.

The polyploidy-induced increase in the number of chloroplast genomes is one of the processes that overcomes the imbalance of cytonuclear interactions and is one of the most studied (Coate, Farmer, et al., 2020; Fernandes Gyorfy et al., 2021; Oberprieler et al., 2019). In this work, it was observed that the imbalance in cytonuclear complexes in the *Festuca-Lolium* group is fully compensated by an increase in the copy number of chloroplast genomes. The increase we observed was pronounced and continuous. The genomes of both neopolyploids (C0) and established polyploids (C8) were examined, and it was observed that the number of genome copies increased from 2.1-fold in neopolyploids to 2.5-fold in subsequent generations compared to the diploids. This finding is consistent with the results of Fernandes Gyorfy et al. (2021), who demonstrated a two to three-fold increase in tetraploid Aegilops and Arabidopsis compared to diploids, immediately after WGD. In contrast, studies of *A. thaliana* and *Leucanthemum* have not demonstrated full compensation through an increase in the number of chloroplast genome copies (Coate et al., 2020; Oberprieler et al., 2019). This suggests that different species may cope differently with this change in the balance of cytonuclear interactions.

Another way in which a plant can cope with a change in stoichiometry in cytonuclear complexes after WGD is by altering gene expression through up- or down-regulation of involved genes. The results of this study indicate that there was no significant up- or down-regulation of nuclear or chloroplast genes in the pure species.

This finding is consistent with the results of Grover et al. (2022), who found no evidence of compensation for changes in the ratio of the amount of genetic information by changes in expression. In contrast to this, Coate et al. (2020) observed a reduction in the expression of nuclear genes targeted to chloroplasts. This is consistent with the results we observed in established polyploids, which indicates that the trend may change in subsequent generations. At last, we observed significant up-regulation of chloroplast-encoded genes in hybrid species. The same results were observed by Forsythe et al., (2022), which provides an evidence that allopolyploidization affects the ability to cope with disturbance in cytonuclear interactions. This is not limited to the effects of polyploidization but also encompasses the influence of different inheritance patterns of nuclear and organellar genomes.

Taken together, the results of the microscopy and gene expression analysis indicate that there is a difference between *Arabidopsis* pure species and the hybrids in the way they respond to polyploidization. In both *Arabidopsis thaliana* and *lyrata*, we observed an increase in the number of chloroplasts while chloroplast-encoded gene expression was not significantly altered. On the other hand, in the hybrids, we did not observe a significant change in the number of chloroplasts, but the stoichiometry seems to be balanced by an increase in the expression of the chloroplast-encoded genes. However, the chloroplast genome copy number analysis for all groups needs to be finished to get the full picture of the cytonuclear balance restoration after WGD in this plant system.

# 7 Conclusion

The objective of this thesis was to examine the intracellular changes that occur following WGD in cell morphology, chloroplast genome copy number, and the expression of selected genes involved in cytonuclear interactions. I observed these in autopolyploids and allopolyploids of two models: *Festuca-Lolium* group (*F. pretensis* and *L. multiflorum*) and *Arabidopsis* group (*A. thaliana* and *A. lyrata*).

The thesis demonstrated that in autopolyploids, the imbalance in cytonuclear interactions, which is an outcome of the WGD, was fully compensated by an increase in the number of chloroplasts, accompanied by an increase in the copy number of chloroplast genomes. The analysis of gene expression did not reveal any significant differences between ploidy except for subsequent generations of tetraploid *Arabidopsis thaliana* where the expression of nuclear-encoded genes was decreased in the tetraploids compared to the diploids and therefore may also contribute to the balance restoration. In allopolyploids, we observed a different mechanism of the cytonuclear stoichiometry restoration where the major contributor seemed to be an alteration of the transcript levels of chloroplast encoded genes. Further experiments are needed, however, to confirm this result.

Understanding the cytonuclear interactions, in particular in polyploids and hybrids will be useful not only to the research community but also to plant breeders as hybridization and polyploidization are commonly used in agriculture. Getting more insight into how they work genetically can improve the process of breeding new crop species and help us cope with the changing environment.

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# 9 Abbreviations

**Abbreviation 1:** The results of cell morphology analysis

| Summary | | Number of chloroplasts | Cell volume [µm$^3$] | Chloroplast volume [µm$^3$] | Nucleus volume [µm$^3$] |
|---|---|---|---|---|---|
| **A. thaliana 2x** | **Average** | 30,4 | 8630 | 260 | 86 |
| | **SE** | 0,5 | 415 | 9 | 2 |
| **A. thaliana 4x C2** | **Average** | 44,3 | 16612 | 443 | 75 |
| | **SE** | 0,9 | 774 | 16 | 3 |
| **A. thaliana 4x C4** | **Average** | 59,9 | 14097 | 226 | 74 |
| | **SE** | 0,8 | 554 | 8 | 2 |
| **A. lyrata 2x** | **Average** | 41,3 | 17603 | 204 | 108 |
| | **SE** | 0,8 | 743 | 9 | 4 |
| **A. lyrata 4x** | **Average** | 53,9 | 15993 | 283 | 93 |
| | **SE** | 0,9 | 686 | 16 | 2 |
| **Hybrid 2x** | **Average** | 53,1 | 14258 | 224 | 92 |
| | **SE** | 0,9 | 581 | 9 | 2 |
| **Hybrid 4x** | **Average** | 56,7 | 20126 | 309 | 95 |
| | **SE** | 1,0 | 926 | 14 | 2 |