The relative copy number of mitochondrial and chloroplast DNA in young and mature leaves of different grape varieties

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Abstract. The copy number of DNA matrices of subcellular organelles (plants) can serve as an indicator of the intensity of photosynthesis and oxidative phosphorylation processes. We assessed the relative copy numbers (RCN) of mitochondrial and chloroplast DNA in young and mature leaves of three grape varieties: 'Traminer Pink', 'Chardonnay', and 'Syrah', grown under field conditions. Leaf samples (5–10 mg) were randomly selected from each group of plants for subsequent total DNA extraction. The qRT-PCR reaction was performed using LightCycler 480 SYBR Green I Master Mix (LifeScience, Roche) and a LightCycler 96 Automatic Analyzer (Roche Life Science). The relative copy numbers of the NAD1 gene (mitochondrial DNA) and rps16 gene (chloroplast DNA) were determined using the GAPDH gene (chromosomal DNA) as a reference. Quantitative assessment was conducted using the $2^{-\Delta Ct}$ и $2^{-\Delta\Delta Ct}$ algorithms. It has been established that the relative copy number (RCN) values of chloroplast and mitochondrial DNA vary and depend on the grape variety and leaf maturity. RCN of chloroplast and mitochondrial DNA is significantly higher in mature grape leaves of all studied varieties, indicating a higher intensity of photosynthesis and oxidative phosphorylation in mature grape leaves compared to young leaves. When assessing the MacroErgic Balance (MEB) indicator, it can be concluded that from 2 to 4% of the energy obtained in chloroplasts through photosynthetic processes is used for the production of macroergic compounds in the mitochondria of various grape varieties in both young and mature leaves. The experimental scheme we have developed can be successfully used as a testing system to assess the potential yield of various grape varieties.

1 Introduction

Plant cells contain two organelles, surrounded by double membranes (plastids and mitochondria), each of which has its own genome, similar to the genomes of bacterial cells [1]. Chloroplasts and mitochondria are of vital importance for harnessing light energy, producing energy through oxidative phosphorylation, and maintaining the homeostasis of plant cells. The mitochondrial and chloroplast DNA of grapes have a circular structure with

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sizes of 773,279 bp (RefSeq: NC_012119.1) and 160,928 bp (RefSeq: NC_007957.1), respectively. One of the peculiarities of the DNA of these subcellular organelles is that it exists in a large number of copies, both in mitochondria and chloroplasts, which significantly varies in different tissues and during the development of plants. Variations in the number of organelle DNA copies reflect the extent of their functional load since DNA serves as a translational-active matrix. It is believed that the connection between the metabolic needs of the cell and the ability of mitochondria and chloroplasts to meet these needs acts as a selective force regarding the number of organelles and genome copies per organelle [2].

Both mitochondrial and chloroplast DNA in plants exist as circular molecules, preserving their inherited bacterial architecture [3]. Unlike nuclear DNA in diploids, which has two copies of each gene per cell, each subcellular organelle (mitochondria and chloroplasts) contains several copies of DNA, and each plant cell has numerous mitochondria and chloroplasts. Each plant cell possesses numerous complete copies of mtDNA and cpDNA, and this concept of copy number variation within subcellular organelles is widely used in studies of their functions [4]. Numerous studies have demonstrated the existence of a regulatory system to maintain organelle DNA copy numbers in plants, where copy numbers depend on the age and physiological activity of plant tissues [5]. However, little is known about changes in DNA copy number levels within subcellular organelles of different grape varieties at different stages of leaf development (young and mature leaves).

Solar energy is efficiently utilized by plants to store energy and synthesize organic substances. This process, known as photosynthesis, is localized in organelles called chloroplasts. The quantity of chloroplast DNA (transcriptionally active templates) reflects the intensity of these organelles' work [6]. Therefore, in our study, we examined the Relative Copy Number (RCN) of chloroplast DNA in young and mature grape leaves, assessed based on their relative copy numbers.

The goal of our research was to investigate potential quantitative patterns in the relative copy numbers of DNA within subcellular organelles (mitochondria and chloroplasts) in young and mature leaves of different grape varieties ('Chardonnay,' 'Traminer Pink,' and 'Syrah') grown in field conditions, as indicators that potentially influence their productivity.

2 Materials and Methods

Sample collection and subsequent analysis were conducted in July 2023 on grapevine plants (*Vitis vinifera* L.) in the collection vineyard of the V.I. Vernadsky Crimean Federal University (Republic of Crimea, Simferopol). On July 6, 2023, young and mature fresh leaf samples were collected from grapevines of the 'Traminer Pink,' 'Chardonnay,' and 'Syrah' varieties in field conditions.

For each leaf sample (5–10 mg), total DNA extracts were prepared [7]. Within the study of the 'Traminer Pink,' 'Chardonnay,' and 'Syrah' varieties, molecular genetic research was conducted on each of the six samples of mature and young leaf samples from each variety.

Quantitative RT-PCR was performed in a 25 µl reaction mixture consisting of 12.5 µl LightCycler 480 SYBR Green I Master Mix (LifeScience, Roche, Penzberg, Germany), 5 ng DNA (5 µl), 5.5 µl of water, and 1 µl of corresponding primers (forward and reverse, 0.33 µM). RT-PCR was carried out using an automatic analyzer, Light-Cycler 96 (Roche Life Science), with the following program: initial denaturation at 95°C for 5 minutes (1 cycle), followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 25 seconds, and extension at 72°C for 25 seconds. The determination of RCN for the NAD1 gene (mitochondrial DNA) and RPS16 gene (chloroplast DNA) was carried out using the GAPDH gene (chromosomal DNA) as a reference [8]. Quantitative assessment was performed using the 2-DCt and 2-DDCt algorithms [9].

The obtained RCN values for organelles were evaluated as the relative quantity of transcriptionally active organelle matrices (which do not coincide with the number of organelles) per cell. Therefore, the data were recalculated per diploid genome. The RCN ratios of chloroplast and mitochondrial DNA (RCN) allow us to assess the relationship between the intensities of light energy accumulation during photosynthesis (RCN cpDNA) and its consumption in oxidative phosphorylation to produce macroergic compounds (RCN mtDNA). We expressed this relationship in the form of an index:

$$\text{Energy Balance (EB)} = \quad \text{RCN(chlDNA)RCN(mtDNA)},$$

that reflects the ratio between transcriptionally active chlDNA and mtDNA matrices.

For an indirect assessment of what percentage of the energy produced by chloroplasts is consumed in mitochondria to produce macroergic compounds, we used the following formula:

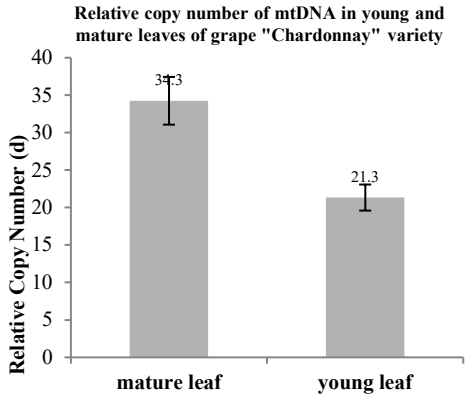
$$\text{MacroErgic Balance (MEB)} = \quad 100\text{-(RCNchlDNA-RCNmtDNARCNclDNA}\times 100\ \%$$

Table 1. Primers Used in the Study

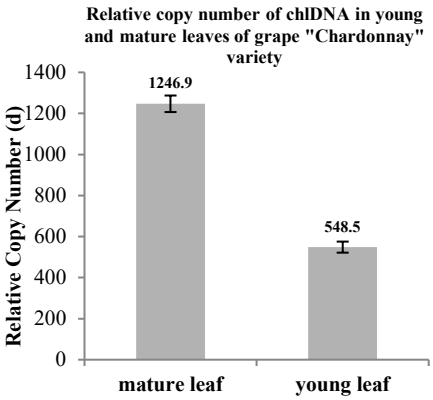
Primer name	Nucleotide sequence, 5′→3′	Amplicon length	Tm (^{0}C)	Cellular compartment
***GAPDH*_F**	CGA CAG TGT TCA CGG TCA GT	**85**	**60**	Nuclear DNA
***GAPDH*_R**	GGT GAC TGG CTT CTC ACC AA			
***RPS16*_F**	CGG ATC ATA AAA ACC CAC TTT CCG	81	60	Chloroplast DNA
***RPS16*_R**	GCC GTC TAT CGA ATC GTT GC			
***NAD1*_F**	GGC TCA TTC TCC AAA CGG GA	73	60	Mitochondrial DNA
***NAD1*_R**	CCT ATG GCC GAT CTG TCA CC			

Statistical analysis was performed using Statistica 13.3.0 software (TIBCO Statistica, 2017) with default parameters. The statistical significance of differences in the examined datasets was assessed using the Student's t-test, and differences with a p-value of less than 5% were considered statistically significant.

3 Results and discussion



a) Relative copy number of mitochondrial DNA (mtDNA)

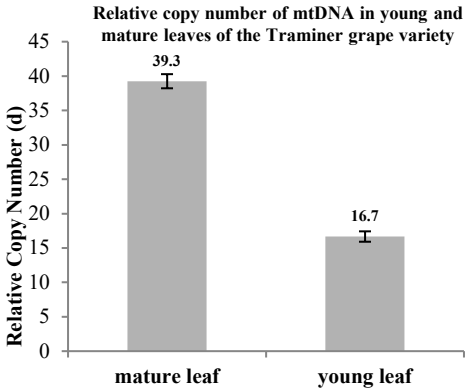


b) Relative copy number of chloroplast DNA (chlDNA)

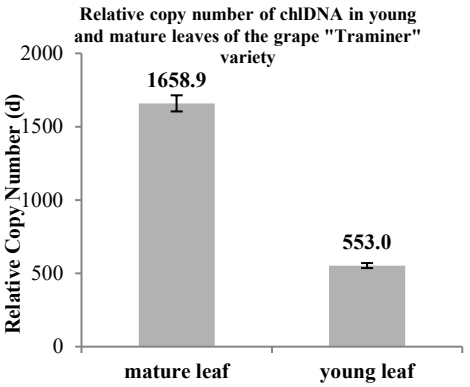
Fig. 1. RCN (Relative Copy Number) of mitochondrial DNA (mtDNA) and chloroplast DNA (chlDNA) in young and mature leaves of 'Chardonnay' grapevines grown in field conditions. Mean values are presented. GAPDH (nuclear DNA) was used as a reference gene for comparison. The data are expressed relative to the diploid genome (d).

As evident from our experimental data presented in Figure 1a, the mean arithmetic value of the relative copy number of mitochondrial DNA (mtDNA) in mature grapevine leaves was 34.3 copies per diploid genome (per cell), while in the tissues of young leaves, the corresponding value was 21.3 copies. Thus, the relative copy number of mtDNA in mature 'Chardonnay' grapevine leaves is 1.6 times higher than the equivalent value for young leaves. These differences are statistically significant at a p-value of 0.003% (T-Student Test, Homoscedastic) and indicate a higher level of oxidative phosphorylation in mature grapevine leaves compared to young leaves.

The relative copy number of chloroplast DNA (chlDNA) in mature 'Chardonnay' grapevine leaves (Figure 1b) amounted to 1246.9 copies per diploid genome, while in the tissues of young leaves, the corresponding value was 548.5 copies. These data suggest that the relative copy number of chlDNA in mature grapevine leaves is 2.3 times higher than the equivalent value for young leaves. These differences are statistically significant at a p-value of 0.001% (T-Student Test, Homoscedastic) and indicate a higher level of photosynthetic processes in mature grapevine leaves compared to young leaves. It can be concluded that in mature 'Chardonnay' grapevine leaves grown in field conditions, the level of photosynthetic processes and oxidative phosphorylation is nearly 2 times higher than in young leaves with a high degree of statistical significance.



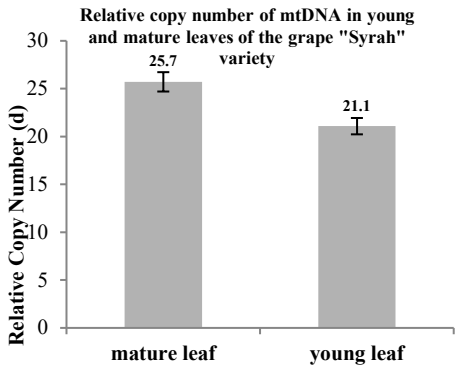
a) Relative copy number of mitochondrial DNA (mtDNA)



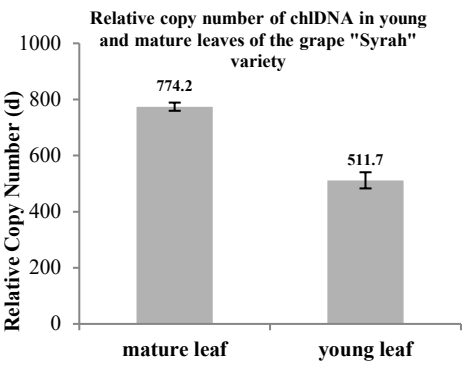
b) Relative copy number of chloroplast DNA (chlDNA)

Fig. 2. RCN (Relative Copy Number) values of mitochondrial DNA (mtDNA) and chloroplast DNA (chlDNA) in young and mature leaves of 'Traminer Pink' grapevine grown under field conditions. Mean arithmetic values. GAPDH is used as the reference gene for comparison, and the data are adjusted for diploid genome (d).

Based on our experimental data presented in Figure 2a, the mean arithmetic value of the relative quantity of mitochondrial DNA copies (Figure 2a) in mature grapevine leaves of the 'Traminer Pink' variety was 39.3 copies per diploid genome, while the corresponding figure in the tissues of young leaves was 16.7 copies per diploid genome.



a) Relative copy number of mitochondrial DNA (mtDNA)



b) Relative copy number of chloroplast DNA (chlDNA)

Fig. 3. RCN values of mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) in young and mature leaves of 'Syrah' grapevines grown under field conditions. Mean arithmetic values. GAPDH was used as the reference gene for comparison (nuclear DNA). Data are presented in diploid genome equivalents (d).

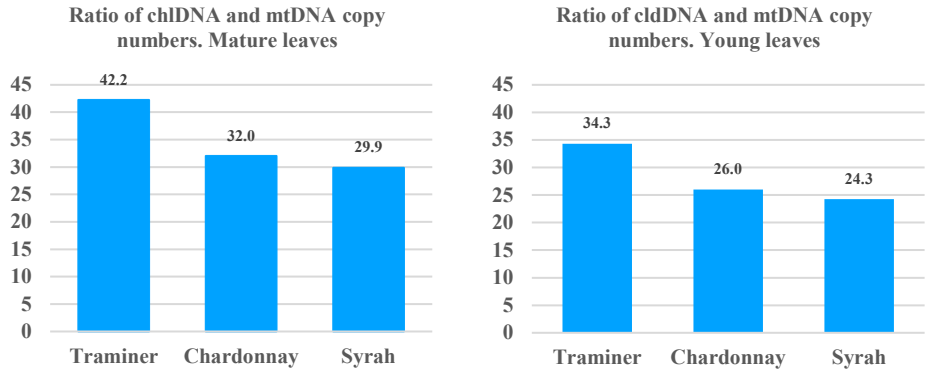
Thus, the relative quantity of mtDNA in mature grapevine leaves of the 'Traminer Pink' variety exceeded that in young leaves by a factor of 2.4, and these differences were statistically significant at a p-value of 0.001% (T-Student Test, Homoscedastic). This suggests a higher level of oxidative phosphorylation in mature grapevine leaves of the 'Chardonnay' variety compared to young leaves.

Relative copy number of chloroplast DNA (cpDNA) in mature grapevine leaves of the 'Traminer Pink' variety (Figure 2b) was found to be 1658.9 copies per diploid genome, while

a similar measure in young leaves was 553.0 copies per diploid genome. These data indicate that the relative copy number of cpDNA in mature grapevine leaves of the 'Traminer Pink' variety is 3 times higher than that in young leaves. These differences are statistically significant with a p-value of 0.001% (T-Student Test, Homoscedastic) and suggest a higher level of photosynthetic processes in mature leaves of the 'Traminer Pink' grapevine compared to young leaves. It can be concluded that in mature leaves of 'Traminer Pink' grapevines grown under field conditions, the levels of photosynthetic processes and oxidative phosphorylation exceed those in young leaves by 3 and 2 times, respectively, with a high degree of statistical significance.

In Figure 3, our experimental data demonstrate that the mean relative quantity of mitochondrial DNA (mtDNA) (Figure 3a) in mature grapevine leaves of the 'Syrah' variety is 25.7 copies per diploid genome, whereas a similar measure in young leaves is 21.1 copies per diploid genome. Thus, the relative copy number of mtDNA in mature leaves of the 'Syrah' grapevine exceeds that in young leaves by a factor of 1.2. These differences are statistically significant with a p-value of 0.003% (T-Student Test, Homoscedastic), indicating a 20% increase in oxidative phosphorylation in mature 'Syrah' grapevine leaves compared to young leaves.

The relative copy number of chloroplast DNA (cpDNA) in mature grapevine leaves of the 'Syrah' variety (Figure 3b) was 774.2 copies per diploid genome, while a similar measure in young leaves was 511.7 copies per diploid genome. These data indicate that the relative copy number of cpDNA in mature 'Syrah' grapevine leaves is 1.5 times higher than that in young leaves. These differences are statistically significant with a p-value of 0.003% (T-Student Test, Homoscedastic), suggesting approximately a 50% higher level of photosynthetic cpDNA-matrices in mature grapevine leaves of the 'Syrah' variety compared to young leaves. If we assume that the presence of translational-active DNA matrices is directly proportional to the functional activity of the corresponding organelles, it can be concluded that in mature 'Syrah' grapevine leaves grown under field conditions, photosynthetic processes and oxidative phosphorylation are 50% and 20% higher, respectively, than in young leaves, with a high degree of statistical significance (p-value=0.003%, T-Student Test, Homoscedastic).



a) Mature leaves b) Young leaves

Fig. 4. Data on EB indicators. Comparative ratios of RCN chloroplast DNA (cpDNA) to RCN mitochondrial DNA (mtDNA) in young and mature grapevine leaves of the 'Syrah,' 'Traminer Pink,' and 'Chardonnay' varieties grown under field conditions.

Results of testing young and mature leaves of three grapevine varieties for RCN chloroplast DNA to RCN mitochondrial DNA ratios (EB indicator) are presented in Figures

№. 4a and 4b. As seen from the data in Figures №. 4a and 4b, the highest prevalence of chloroplast DNA-matrix over mitochondrial DNA-matrix is observed in mature leaves compared to young leaves. This observation is consistent across all the grapevine varieties studied: 'Traminer Pink,' 'Chardonnay,' and 'Syrah,' grown under field conditions. It's worth noting the heterogeneity of this pattern among different grapevine varieties: the highest chloroplast DNA-matrix prevalence is found in 'Traminer Pink,' where mature leaves have 42 times more cpDNA-matrix than mtDNA-matrix, and in young leaves, this ratio is 34.3. The lowest ratio of cpDNA-matrix to mtDNA-matrix is observed in 'Syrah,' with mature leaves at 29.9 and young leaves at 24.3. According to literature data, 'Syrah' is categorized as a variety with low yields [10], and the indicator we used may reflect potential productivity differences among grapevine varieties, which will be further investigated.

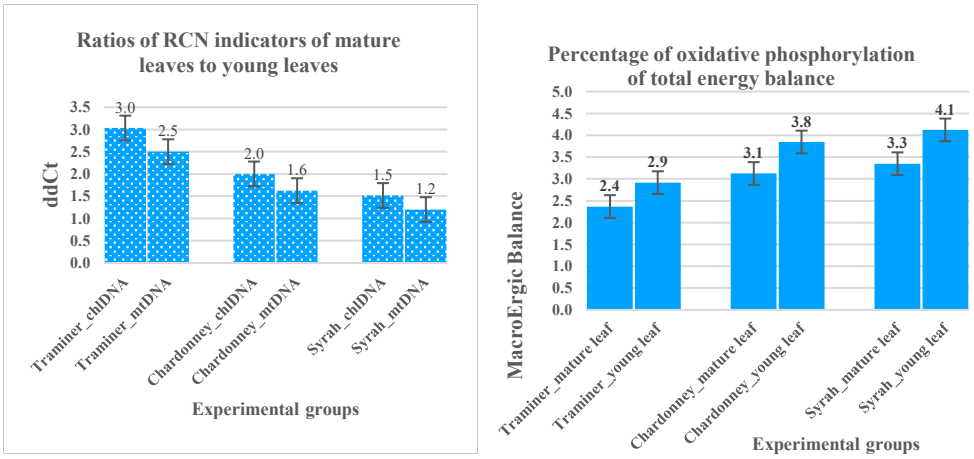


Fig. 5. Energy balance indicators in different experimental grapevine groups for the 'Syrah,' 'Traminer Pink,' and 'Chardonnay' varieties grown under field conditions. Mature and young leaves.

In Figure № 5a, we visually demonstrate the indicators of increased translational-active DNA matrices for the 'Syrah,' 'Traminer Pink,' and 'Chardonnay' grapevine varieties grown under field conditions, expressed as the ratio of relative DNA copy numbers in mature leaves to young leaves. This figure clearly shows a multiple increase in translational-active DNA matrices of organelles (chloroplasts and mitochondria) in mature leaves compared to young leaves. These differences are statistically significant at a p-value of 0.05% (T-Student Test, Homoscedastic).

Figure № 5b presents data on the MacroErgic Balance, which represents the percentage ratio of translational-active mtDNA matrices (oxidative phosphorylation) to translational-active cpDNA matrices (photosynthesis) for the 'Syrah,' 'Traminer Pink,' and 'Chardonnay' grapevine varieties in mature and young leaves. A clear tendency of higher oxidative phosphorylation levels in young leaves compared to mature leaves is ticeable. This is likely due to the need to expend part of the energy obtained during photosynthesis for the synthesis of macro-energetic compounds required for cell proliferation processes. Using the MEB algorithm, we determined that all the grapevine varieties included in this study, both in young and mature leaves, use approximately 2 to 4% of the energy obtained during photosynthesis for the production of macro-energetic compounds in mitochondria.

4 Conclusions

1. The relative copy number (RCN) values of chloroplast DNA are variable and dependent on the grapevine variety and leaf maturity.

2. In our study, RCN of chloroplast DNA ranged from 1659 copies of chloroplast DNA in mature leaves of 'Traminer Pink' grapevine to 774.2 in mature leaves of 'Syrah' grapevine.

3. RCN of chloroplast DNA in young leaves significantly lagged in quantitative terms behind the corresponding values in mature leaves, ranging from 553 copies in 'Traminer Pink' grapevine to 512 RCN in 'Syrah' grapevine. This suggests a higher level of photosynthetic processes in young leaves compared to mature leaves.

4. The relative copy number (RCN) of mitochondrial DNA is also variable, showing varietal specificity and dependence on leaf maturity. RCN of mitochondrial DNA ranged from 39 copies in 'Traminer Pink' grapevine to 25.7 in 'Syrah' grapevine in mature leaves.

5. RCN of mitochondrial DNA in young leaves ranged from 21 copies in 'Chardonnay' and 'Syrah' grapevines to 16.7 in 'Traminer Pink' grapevine.

6. Our results indirectly indicate a higher intensity of oxidative phosphorylation processes in the tissues of mature leaves compared to young leaves.

7. When assessing the MacroErgic Balance (MEB) indicator, it can be concluded that 2% to 4% of the energy obtained in chloroplasts through photosynthetic processes is expended on the production of macro-energetic compounds in the mitochondria of young and mature leaves of different grapevine varieties. Moreover, more energy obtained during photosynthesis is expended on the synthesis of macro-energetic compounds in young grapevine leaves compared to mature leaves. This is likely necessary to intensify cell division processes.

The experimental scheme developed by us can be successfully used as a test system to assess the potential yield of different grapevine varieties.

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