Changes in the relative copy numbers of chloroplast and mitochondrial DNA in the leaves of *Vitis vinifera* L. after high-temperature treatment in vitro

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Abstract. In the context of global warming, studying the consequences of increased temperature on agricultural crops becomes important for predicting the short- and long-term impacts on productivity. The effects of elevated temperature on grapevine plants lead to increased yield losses in viticulture. Micropropagated grapevine plants of the 'Chardonnay' variety were grown *in vitro* on MS medium and subjected to heat treatment at 45°C for 120 minutes. The control group of plants was not exposed to heat treatment. The levels of relative copy numbers of chloroplast and mitochondrial DNA were determined in leaf tissues of all plant groups using the RT-PCR method 30 days after heat treatment. In the group of plants subjected to heat treatment, statistically significant ($p>0.05$) reductions in the relative copy numbers of mitochondrial and chloroplast DNA were observed compared to the control group, with a decrease of over 30%. The copy number of chloroplast DNA exceeded that of mitochondrial DNA by more than 20 times in both the experimental and control groups. Heat treatment of micropropagated grapevine plants *in vitro* resulted in a closer correlation ($r=+0.86$) in the regulation of activity between these organelles, alongside the decrease in relative copy numbers of both mitochondrial and chloroplast DNA. This study demonstrates the promising use of relative copy numbers of chloroplast and mitochondrial DNA in plant leaves to investigate their potential physiological response to adverse environmental factors.

1 Introduction

Plant cells, including grapevines, contain multiple copies of mitochondrial and chloroplast genomes, encoding key components of the mitochondrial electron transport chain and photosynthesis, respectively. Changes in mitochondrial DNA (mtDNA) and chloroplast DNA through inactivating genetic mutations or alterations in their copy numbers within a cell can modify the intensity of mitochondrial respiration and photosynthetic processes, which has drawn the attention of researchers [1, 2]. Peak temperature stresses at critical stages

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of grapevine development have detrimental effects on its growth [4] and influence photosynthesis [5]. Characteristics of chromosomal DNA serve as a stable indicator for each species and are independent of functional loads, while the relative copy numbers of DNA in intracellular organelles (mtDNA-OKK and cpDNA-OKK) in plants act as biomarkers reflecting the intensification or suppression of their function or indicating dysfunction. Therefore, the measurement of mtDNA-OKK and cpDNA-OKK can be a valuable and accessible biomarker for assessing changes in the activity levels of these organelles [6]. CpDNA and mtDNA are expected to be abundant in leaves, where they form punctate structures known as nucleoids, which change their morphology and quantity depending on the physiological state of the plant. The copy number of DNA in organelles directly affects the level of RNA they express, making it a marker for the intensity of these organelles' work in plants [7].

Given the economic and cultural significance of vineyards, it is necessary to study the mechanisms of abiotic factors' impact on essential cellular organelles and develop effective markers for evaluating and, if possible, minimizing the degree of destructive influence from adverse factors.

2 Materials and Methods

At the initial stage of the model experiment, all explants of *Vitis vinifera* L. 'Chardonnay' were cultivated on a Murashige and Skoog medium (MS) with the addition of 30 g/L sucrose, 0.2 mg/L indole-3-acetic acid (IAA), and 2 mg/L 6-benzylaminopurine (6-BAP) [8]. Each of the experimental containers with a nutrient medium contained 10 grape explants. The experimental containers with the nutrient medium and explants were then divided into two groups:

1. Experimental group of plants subjected to heat treatment at 45°C for 120 minutes.
2. Control group of plants not subjected to heat treatment.

After the completion of the heat treatment of the grapes at 45°C for 120 minutes, both experimental groups were combined, and the plants were incubated at a temperature of 23 ± 1°C, with a 16-hour photoperiod and illumination from 40-watt cold white fluorescent lamps with an intensity of 105–115 μmol PPFD/m²/s (PPFD = photosynthetic photon flux density) for 30 days. From each group of plants (control and post-heat treatment), fourteen leaf blade samples (5–10 mg) were randomly selected for subsequent extraction of total DNA [8].

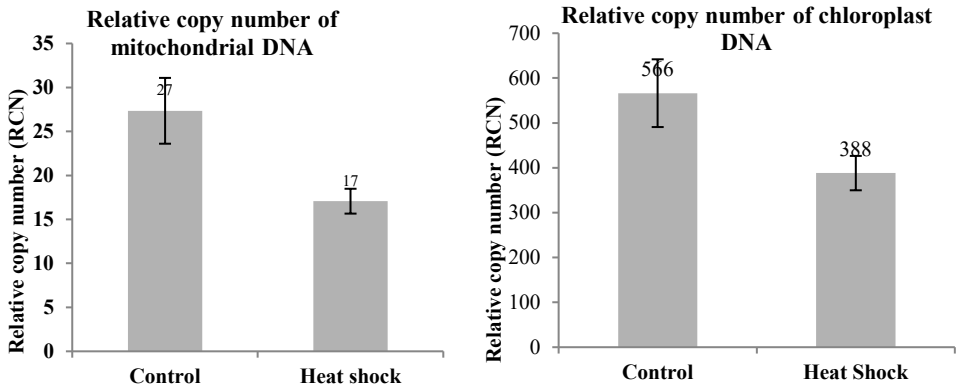
Quantitative RT-PCR was performed in a 20 μL reaction mixture, consisting of 10 μL LightCycler 480 SYBR Green I Master Mix (Life Science, Roche), 5 ng of DNA (5 μL), 3 μL of water, and 1 μL of respective primers (forward and reverse, 0.33 μM). RT-PCR was conducted using an automated analyzer, LightCycler 96 (Life Science, Roche), 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 relative copy numbers of NAD1 (mitochondrial DNA) and rps16 (chloroplast DNA) genes were determined using the GAPDH gene (chromosomal DNA) as a reference. Quantitative assessment was performed using the 2-DCt and 2-DDCt algorithms [9].

Table 1. Characteristics of 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 carried out using Statistica 13.3.0 software (TIBCO Statistica, 2017) with default parameters. The statistical significance of differences between datasets was assessed using Student's t-tests (Homoscedastic and Heteroscedastic) and the Fischer's F-test.

3 Results and discussion



a) b)

Fig. 1. Mean relative DNA copy numbers of mitochondria (a) and chloroplasts (b) compared to the GAPDH genome in grape leaves of the 'Chardonnay' variety following limited temperature exposure at 45°C for 120 minutes (heat treatment) and in the control group of plants.

Statistical analysis of morphometric data demonstrated no differences between the control and experimental plant groups in terms of survival rate, plant height, and the number of leaves. This confirms the correctness of the selected heat treatment conditions.

As depicted in our experimental data presented in Figure 1, the relative copy number of chloroplast DNA in the leaves of grape plants in the control group was 566 copies, while 30 days after the temperature treatment (45°C, 120 minutes), it decreased to 388 copies (Figure 1b). Our data clearly showed a statistically significant 31.4% reduction in the relative copy number of chloroplast DNA in grape leaves after the temperature treatment, compared to control plants. These differences were statistically significant at a 2.5% significance level when using Student's t-test. Analysis of variance using Fisher's F-test revealed statistically significant differences between the datasets at a 1% significance level.

The relative copy number of mitochondrial DNA in the leaves of grape plants in the control group was 27.3 copies, whereas 30 days after the temperature treatment (45°C, 120 minutes), it decreased to 17.1 copies (Figure 1a). Our data demonstrated a 37.6% reduction in the relative copy number of mitochondrial DNA in grape leaves after the temperature treatment compared to control plants. These differences were statistically significant at a 1% significance level when using Student's t-test. Analysis of variance using Fisher's F-test also revealed statistically significant differences between the datasets at a 1% significance level, considering the Bonferroni-corrected significance level.

Our experimental data indicate that even a single heat treatment of grape microplants under in vitro conditions results in a statistically significant reduction in the relative copy number of mitochondrial DNA in microplant leaves, leading to the inhibition of oxidative phosphorylation even after 30 days under normal conditions.

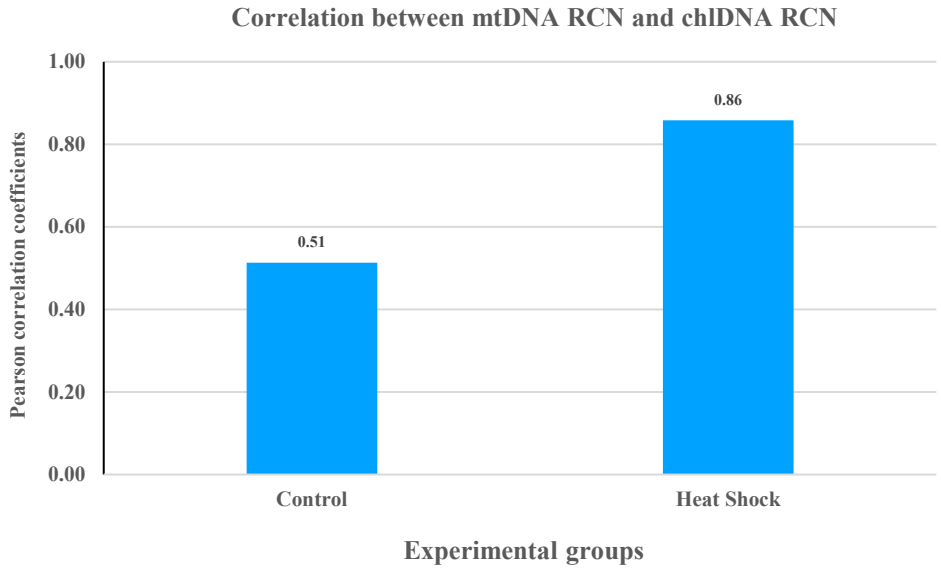


Fig. 2. Pearson correlation coefficients for the relative copy numbers of chloroplast and mitochondrial DNA in grape leaves (following temperature treatment at 45°C for 120 minutes and in the control group).

Plants obtain most of their energy from sunlight captured in chloroplasts, which is used for various synthetic and energy exchange processes and other energy-requiring activities in plants, either directly or indirectly. However, in non-green parts of plants or in the dark, energy acquired by plant cells during photosynthesis is derived from oxidative phosphorylation, which takes place in mitochondria [10]. Therefore, in this section of our study, we assessed the correlation between the relative copy numbers of mitochondrial and chloroplast DNA in control microplants and in microplants after heat treatment.

Our data demonstrated a statistically significant increase in the correlation between the relative copy numbers of mitochondrial and chloroplast DNA in grape leaves following the temperature treatment (45°C, 120 minutes) after 30 days. For the experimental group "Heat Treatment," the correlation value we obtained (r=+0.86) was statistically significant for a 95% confidence interval, while in the experimental group ("Control"), these values demonstrated a weakly positive correlation (r=+0.51). This suggests that temperature treatment of grape microplants under in vitro conditions, alongside the reduction in the relative copy numbers of both mitochondrial and chloroplast DNA, leads to a tighter positive relationship in regulating the activities of these organelles (Figure 2).

Our experimental data revealed a predominance of the relative copy number of chloroplast DNA over mitochondrial DNA by approximately 22-fold. Statistically significant differences were observed between the control and experimental data for the ratios of relative copy numbers of chloroplast to mitochondrial DNA in grape leaves after 30 days (p=0.028%), determined using Fisher's F-test. The mean values of these series were not statistically different: 22.5 and 22.4, respectively.

Our experimental data indicate that heat treatment of grape microplants under in vitro conditions, in the context of reduced relative copy numbers of both mitochondrial and chloroplast DNA, leads to the predominance of chloroplast DNA copy numbers over mitochondrial DNA.

4 Conclusions

1. Plants possess a mechanism for regulating organelle copy numbers depending on their functional load and in response to abiotic stressors [10].

2. Our findings clearly demonstrated a statistically significant reduction in the relative copy number of chloroplast DNA in grape leaves after the temperature treatment (45°C, 120 minutes) compared to control plants, even 30 days after exposure. This indicates that even a single heat treatment of grape microplants under in vitro conditions results in a statistically significant decrease in the relative copy number of chloroplast DNA in leaves, thereby inhibiting photosynthesis processes even after 30 days following the adverse exposure.

3. Our data also showed a statistically significant reduction in the relative copy number of mitochondrial DNA in grape leaves after the temperature treatment (45°C, 120 minutes) compared to control plants, even 30 days after the exposure. This demonstrates that a single heat treatment of grape microplants in vitro leads to a statistically significant decrease in the relative copy number of mitochondrial DNA in microplant leaves, thereby inhibiting oxidative phosphorylation processes even after 30 days under normal conditions.

4. Indicators of relative DNA copy numbers in plant organelles demonstrate a direct connection with the quantitative characteristics of gene expression and, consequently, their functional activity [10]. Therefore, the patterns we observed regarding changes in the relative copy numbers of mitochondrial and chloroplast DNA after temperature exposure, in comparison to plant morphometric characteristics, have a much more sensitive and predictive nature in terms of potential long-term effects on plant productivity.

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