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PLANT GENETICS

Comparative Analysis of Thermotolerance of Sunflower Chlorophyll Mutants

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Abstract—The influence of high temperatures on sunflower chlorophyll mutants was studied. The tolerance criteria were the level of chromosome aberrations and the mitotic index in the root apical meristem of seedlings, the level of nucleus-free cells in the epidermis of cotyledonous leaves, and the intensity of chlorophyll accumulation after the action of heat shock (HS). In addition, the frequency of plants with an altered content of pigments in M1 and M2 was analyzed. The results indicated that the plastomic mutant en-chlorina-5 is more tolerant to temperature stress as compared to other sunflower lines.

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INTRODUCTION

Tolerance to extreme conditions can depend on the ability of organisms to control the intensity of free radical reactions and the processes of peroxide oxidation of lipids. The participation of plastids and mitochondria in the control of redox homeostasis forms in large part resistance of plants to the action of environmental factors. Functional specificity of plastids and mitochondria determines an increased concentration of free radicals in these organelles as compared to their concentration in nuclei [1–3]. A set of chloroplast and mitochondrial genes that are expressed at a high concentration of reactive oxygen species (ROS) has been formed dirung evolution [3]. This can lead to an increase in the frequency of mutations occurring in chloroplast and mitochondrial DNA. On the other hand, the level of expression of chloroplast and mitochondrial DNA genes directly depends on the redox potential of electron transmitters of the photosynthetic systems of chloroplasts and respiratory enzymes [4]. Such conditions afford ROS concentration-dependent regulation of expression of mitochondrial and plastid genes, promoting a decrease in the production of free radicals in a cell as a whole [3] and thus reducing the frequency of possible mutations in the nuclear genetic material.

Tolerance to extreme influences is most clearly displayed under stress conditions, when the adaptive potentialities of organisms are revealed. The adaptive potential of plant cells is determined by three genetic systems: nuclear, chloroplast, and mitochondrial genes that, on the one hand, can be damaged under the action of extreme factors and, on the other hand, are active participants in stress reactions and can determine resistance or its lack to unfavorable external factors [5–8]. Both nuclear and cytoplasmic mutations are able to change normal reactions of organisms [9, 10]. The purpose of this work was a comparative analysis of three lines of sunflower *Helianthus annuus* L. (initial inbred line 3629 and its nuclear and plastomic mutant derivatives) at different stages of development of plants.

MATERIALS AND METHODS

The study was performed using the inbred line 3629 of sunflower *Helianthus annuus* L. and its plastomic chlorophyll mutant en-chlorina-5 and nuclear chlorophyll mutant n-chlorina-1 obtained by Beletskii et al. [11].

In the cytogenetic experiment, sunflower seeds were soaked for 18 h and then germinated in Petri dishes on moist filter paper. Germinating seeds were exposed to a heat shock (HS) at 47°C during the first 4 h of germination. Then, 24 and 48 h after the HS treatment the rootlets were fixed in a mixture of ethyl alcohol and glacial acetic acid (3 : 1). Using temporary squashed preparations, we determined the mitotic index (MI) in the root apical meristem and the level of chromosome aberrations at the anaphase stage (no less than 8–9 rootlets per variant).

Cells with a damaged nucleus and nucleus-free cells were scored after the treatment in the lower epidermis of cotyledonous leaves of sunflower seedlings aged 8–9 days (from the moment of their emergence). The plants were grown in pots in the dark at room temperature (22–25°C). The soil was usual chernozem. Etiolated experimental plants were exposed to temperature stress (47°C and 53°C) for 6 h. After the exposure, the epidermal films of cotyledonous leaves were removed with a forceps and kept in acetoorsein for 15–20 min. Temporary preparations were made,

Table 1. The levels of chromosome aberrations and proliferative activity in the root meristem of sunflower seedlings after temperature treatment

Variant	Growth time, h	Anaphases total	CA ± *m*	MI ± *m*
		Line 3629		
Control	24	603	2.8 ± 0.45	7.4 ± 0.67
	48	598	3.2 ± 0.5	9.5 ± 0.75
47°C, 4 h	24	593	6.2 ± 0.65***	3.2 ± 0.45***
	48	608	7.3 ± 0.7***	7.4 ± 0.63*
		en-chlorina-5		
Control	24	613	1.7 ± 0.35	10.9 ± 0.8
	48	629	1.95 ± 0.35	10.0 ± 0.77
47°C, 4 h	24	583	5.8 ± 0.51***	7.5 ± 0.69**
	48	600	6.2 ± 0.65***	8.2 ± 0.71
		n-chlorina-1		
Control	24	623	2.3 ± 0.4	6.5 ± 0.64
	48	615	2.7 ± 0.45	9.1 ± 0.74
47°C, 4 h	24	631	6.4 ± 0.65***	1.5 ± 0.03***
	48	627	6.8 ± 0.65***	3.2 ± 0.46***

Note: Significant differences from the control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

and up to 3000 cells per variant were examined among which the proportion of nucleus-free cells was determined [12].

To study the HS influence on the accumulation of pigments, etiolated seedlings at the age of 8–9 days (from the moment of planting) were exposed to elevated temperature (47°C, 50°C, 53°C, and 56°C) in the dark for 2 h. Then the treated and control seedlings grew under conditions of 12-h light day (10 V/m^2); 24 and 48 h after the temperature exposure the content of chlorophylls and carotenoids in cotyledonous leaves of the seedlings was determined [13].

For the field experiment, seeds were soaked in water for 18 h and then germinated in Petri dishes at 26°C. Temperature treatment was carried out during the first 1, 3, or 6 h of germination. In each experimental variant, 150 sprouts were transplanted in the field conditions at optimal terms following the breeding nursery conditions on 10-m plots with a 40 × 60 cm nutrition area. The frequency of plants with an altered content of pigments (in M_1) and the frequency of chlorophyll mutants (in M_2) were estimated.

RESULTS AND DISCUSSION

It was found earlier that elevated temperature (40°C) has different effects on sunflower seedlings of the initial line 3629 and the plastomic mutant en-chlorina-5 [14]. This suggests different thresholds of sensitivity to the action of this external factor. Exposure to a higher temperature (47°C) during the first four hours of germination significantly increases the level of chromosome rearrangements in cells of the initial inbred line 3629 and in the plastomic and nuclear chlorophyll mutants obtained on its basis (Table 1). An increased level of chromosome aberrations is retained during 48 h after the exposure.

However, the lines display differences in the intensity of proliferation of meristematic cells after stress exposure. The heat shock blocks almost completely the division of root meristem cells in the nuclear mutant over a period of 48 h after the end of the treatment; in the initial line 3629, the intensity of cell division restores after 48 h but does not reach the control level. The proliferative activity of cells in the plastomic mutant en-chlorina-5 does not differ from the control values within 48 h after the treatment (Table 1). Thus, the reactions of the nuclear genetic material of root meristem cells of sunflower seedlings of the three lines in response to the action of stressing temperature are the same. However, the plastomic mutant en-chlorina-5 has a more stable cell cycle even at the seedling stage.

The next criterion of sunflower cell tolerance to HS used by us was the level of nucleus-free epidermal cells in etiolated cotyledonous leaves after exposure. As seen in Table 2, the control level of nucleus-free cells in the epidermis of cotyledonous leaves in the line 3629 is 8.7% versus only 1.8% among stoma cells. Similar results were obtained for the plastomic mutant en-chlorina-5. In the nuclear mutant n-chlorina-1, the level of nucleus-free cells in stomata is 5 times higher as compared to that in the initial line 3629.

After the HS, damaged nuclei in all three sunflower lines are predominantly observed in epidermal cells containing only mitochondria, whereas stoma cells

Table 2. The level of nucleus-free cells in the epidermis of sunflower cotyledonous leaves after temperature treatment

Variant	Number of nucleus-free cells, % ± *m*	
	epidermal cells	stoma cells
Line 3629		
Control	8.7 ± 1.1	1.8 ± 1.2
47°C, 6 h	14.0 ± 1.3**	5.0 ± 1.9
53°C, 6 h	20.8 ± 1.5***	14.5 ± 3.1***
en-chlorina-5		
Control	6.7 ± 0.9	2.5 ± 1.4
47°C, 6 h	9.2 ± 1.1	5.8 ± 2.5
53°C, 6 h	19.8 ± 1.4***	11.6 ± 2.9*
n-chlorina-1		
Control	7.3 ± 0.9	8.8 ± 2.4 ^
47°C, 6 h	23.4 ± 1.62*** ^^^	17.5 ± 3.3* ^^^
53°C, 6 h	29.4 ± 1.4*** ^^^	23.8 ± 3.7***

Note: Significant differences from the control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Significant differences from the line 3629: ^$P < 0.05$, ^^^$P < 0.001$.

with chloroplasts show a higher resistance to the action of this factor. In cells of the line 3629 and the plastomic mutant the reactions to HS are similar. However, stoma cells with functionally active chloroplasts in the plastomic mutant are less sensitive to the injurious factor than in the line 3629 (Table 2). In the nuclear mutant n-chlorina-1, the level of epidermal and stoma nucleus-free cells is significantly increased even after exposure to 47°C. Thus, the action of HS induces an increase in the number of nucleus-free cells in cotyledonous leaves. However, while the threshold temperature in the initial line and in the nuclear mutant is 47°C, the proportion of nucleus-free cells in the plastomic mutant increases after exposure to 53°C.

The tolerance of plants at the stage of germination is minimal and does not always correspond to the tolerance of an adult organism. Nevertheless, the action of stress at early stages of vegetation affects a comparatively small number of cell organelles, due to which the probability of phenotypical manifestation of genetic heterogeneity of cytoplasmic DNA-containing organelles increases [15–17]. The stress influence promotes an efficient assortment of organelles and the formation of tissues, organs, and organisms with an abnormal response. Under the action of extreme environmental factors, advantages are acquired by cells in which plastids and mitochondria are adapted to higher concentrations of free-radical products. The sunflower lines under study differ in the activity of antioxidant enzymes and in the intensity of peroxide oxidation of lipids [18].

The degree of sensitivity of plant cells to HS can also be estimated according to the level of chlorophylls [19, 20]. As follows from the analysis of the level of photosynthetic pigments in two lines of wheat *Triticum durum* Desf., plants of the line Adamello are characterized under drought conditions by a reduced level of Chl a, reduced Chl a/b ratio, and a reduced content of carotenoids. At the same time, no changes were detected in the level and composition of pigments in tissues of plants of the drought-resistant line Ofanto [21].

The degree of HS influence on the formation of the photosynthetic apparatus in the three sunflower lines varies. We performed a comparative analysis of the content and accumulation of photosynthetic pigments in etiolated cotyledonous leaves. A gradual accumulation of chlorophylls occurs during 72-h growth in the light in cotyledons of line 3629 seedlings (Fig. 1). The content of pigments in cotyledons of the nuclear mutant n-chlorina-1 after 48-h growth in the light is reduced as compared to that in the line 3629 (Fig. 2). The plastomic mutant en-chlorina-5 exceeds the other lines by the control chlorophyll content in cotyledons of its seedlings grown under artificial illumination (Fig. 3).

HS treatment (47°C, 2 h) of etiolated seedlings of the line 3629 does not influence subsequent accumulation of photosynthetic pigments (Fig. 1). HS at 50°C for 2 h significantly decreases the total Chl a + b content, and higher temperatures (53°C and 56°C) cause a similar effect. Thus, it can be concluded that for line

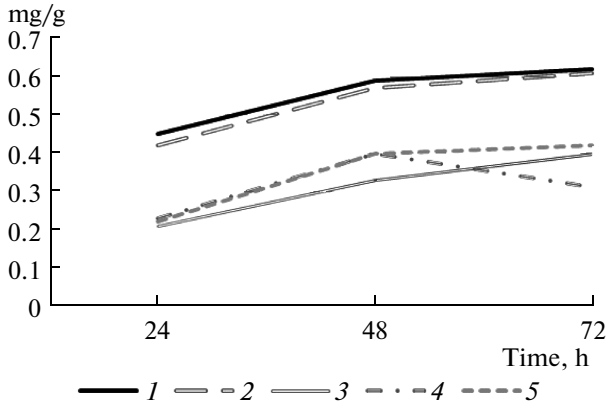
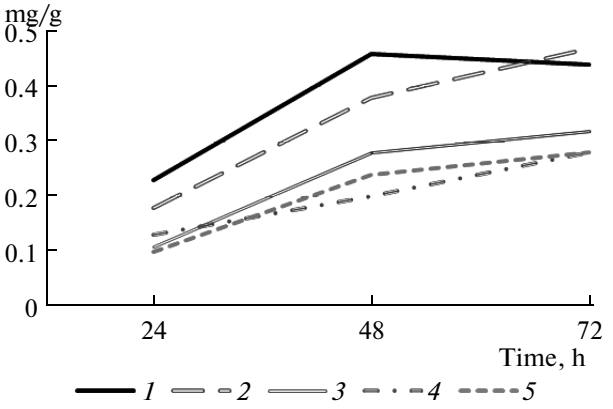


Fig. 1. Dynamics of chlorophyll accumulation in cotyledonous leaves of sunflower line 3629 seedlings after exposure to elevated temperature. *1*, control; *2*, 47°C; *3*, 50°C; *4*, 53°C; *5*, 56°C.



Fig, 2. Dynamics of chlorophyll accumulation in cotyledonous leaves of seedlings of the nuclear mutant n-chlorina-1 after exposure to elevated temperature. *1*, control; *2*, 47°C; *3*, 50°C; *4,* 53°C; *5*, 56°C.

3629 seedlings a threshould temperature is 50°C at which the normal formation of the photosynthetic apparatus is impeded.

Changes in the accumulation of pigments in etiolated cotyledons of the mutant n-chlorina-1 and the line 3629 were noted at temperatures not lower than 50°C (Fig. 2). But after exposure to 53°C and 56°C nearly 30% of n-chlorina-1 seedlings died within 48 h after the end of the exposure. On the whole, the comparison of the effects of HS on etiolated seedlings of the lines 3629 and n-chlorina-1 shows the same tendency of changes in the content of pigments. However, these changes in the mutant are more profound.

Irrespective of the treatment regime, HS had no influence on the intensity of accumulation of pigments in etiolated cotyledons of en-chlorina-5 seedlings (Fig. 3). Thus, the line with a mutation in cytoplasmic DNA displayed tolerance to HS regarding the intensity of synthesis and accumulation of photosynthetic pigments. The fact that the level of chlorophylls a + b in cotyledonous leaves remained unchanged after HS implies integrity of the enzymatic systems involved in the synthesis of chlorophyll and its precursors and in the formation of the ultrastructure of chloroplasts.

The level of thermotolerance is known to be species-specific.

Species-specific differences in the expression of low-molecular chloroplast heat shock proteins after exposure to 45°C were demonstrated in Rhamnaceae [22]. HS in the regime of 55°C, 1.5 h is lethal for 80% of *Sinapis alba* L. seedlings [23]. The authors explained the death of seedlings by the activation of peroxide oxidation of lipids. It was found by us earlier that HS does not activate the processes of peroxide oxidation of lipids in sunflower tissues [18] in contrast to its action in other plant species [24–26]. Synthesis and accumulation of pigments in etiolated sunflower seedlings can also take place at temperatures significantly exceeding the threshold values for other species. For example, it was demonstrated that in etiolated seedlings of *Arabidopsis* temperature treatment at 48°C and 50°C for 30 min completely blocks chlorophyll accumulation and leads to the death of the seedlings [27]. At the same time, it was established that preheating at 38°C induces formation of acquired thermotolerance to subsequent exposure to a higher temperature.

The action of HS during the first hours of germination of sunflower seeds affects their germinating capacity (Fig. 4). HS in the regime of 40°C, 6 h does not practically decrease the germination of seeds of the line 3629 and the plastomic mutant. The level of germination of seeds of the nuclear mutant is significantly reduced (Fig. 4). With raising the temperature up to 45°C, the levels of seed germination for all lines sharply decrease. However, while in the plastomic

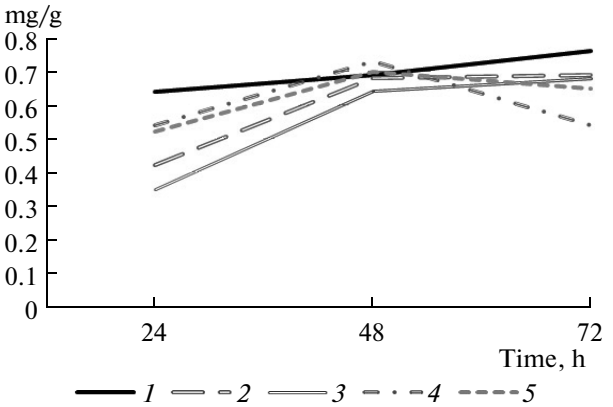


Fig. 3. Dynamics of chlorophyll accumulation in cotyledonous leaves of seedlings of the plastomic mutant en-chlorina-5 after exposure to elevated temperature. *1*, control; *2*, 47°C; *3*, 50°C; *4,* 53°C; *5*, 56°C.

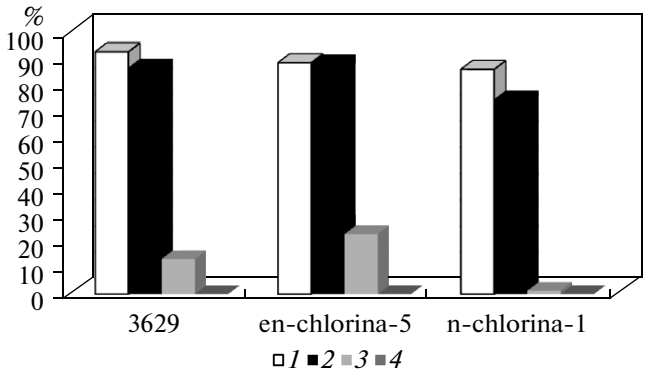


Fig. 4. Germination (%) of sunflower seeds after exposure to elevated temperature. *1*, control; *2*, HS (40°C, 6 h); *3*, HS (45°C, 6 h); *4*, HS (50°C, 6 h).

mutant germination is noted for each fourth seed, in the initial line 3629 it is observed for each sixth seed, and in the nuclear mutant only one of 130 seeds germinates. Moreover, single seedlings of the nuclear mutant died at early stages of vegetation. As shown before, the effect of high temperature (45°C) slows down the growth of plants up to the stage of development of 3–4 pairs of leaves [28]. Exposure to 50°C for 6 h completely inhibits germination of sunflower seeds.

HS at early vegetation stages induces chlorophyll mutations in sunflower. The results of analysis of the genetic effect of HS at 40°C are presented in Table 3. HS in the early hours of germination of sunflower seeds induces the appearance in M_1 of plants with an altered content of pigments in all three lines. In the nuclear mutant n-chlorina-1, HS induces the appearance, in addition to variegated forms, of a great number of lethals (up to 50% of all plants with an altered content of pigments). At the same time, no lethal forms were noted in the plastomic mutant n-chlorina-5.

The analysis of M_2 plants showed that the effect of HS during the first hour of germination did not induce chlorophyll mutations in the line 3629 (Table 3): all variegated M_1 forms turned out to be morphoses and gave a normal green progeny in M_2. A more prolonged action of temperature (3 or 6 h) induces the appearance in M_2 of chlorophyll mutants of the variegated and chlorina types. It should be noted that HS does not induce lethal forms in the line 3629. Lethals in M_2 in the variant with 3-h HS appeared after self-pollination of the variegated form from M_1. All mutants of the chlorina type from M_2 belong to one family and are a progeny of the variegated form from M_1. In this family,

Table 3. The frequency of chlorophyll abnormalities in M_1 and M_2 plants after exposure to elevated temperature (40°C)

Variant	M_1 plants with an altered content of pigments, %	M_2 plants with an altered content of pigments, %			
		variegated	lethals	chlorina	green
Line 3629					
Control	0	0	0	0	
HS (0–1 h)	3.7	0	0	0	
HS (0–3 h)	1.5	0.64 ± 0.45	0.32 ± 0.31	35.2 ± 2.70	
HS (0–6 h)	0.8	2.8 ± 1.37	0	0	
en-chlorinà-5					
Control	0	0	0		0
HS (0–1 h)	2.6	0.45 ± 0.45	0		0
HS (0–3 h)	0.8	0.9 ± 0.87	0		0.9 ± 0.87
HS (0–6 h)	3.8	0	0		0
n-chlorinà-1					
Control	0	0	0		0
HS (0–1 h)	7.0	1.9 ± 1.09	3.8 ± 1.52		0
HS (0–3 h)	4.0	1.5 ± 1.04	0		0
HS (0–6 h)	7.2	0	0		0

chlorina plants did not segregate in M_3 and produced only a light–green progeny, suggesting the nuclear nature of the mutation.

In the plastomic mutant en-chlorina-5, most of variegated forms induced by HS in M_1 proved to be morphoses. In M_2, single variegated chlorina plants and revertants with green leaves were revealed (Table 3). After self-pollination, the variegated chlorina forms segregated into plants of the chlorina and variegated chlorina types in M_3 and M_4. The green revertant forms also segregated into chlorina and green plants. The largest number of chlorophyll mutants in M_2 was revealed in nuclear n-chlorina-1 (Table 3). These mutants were represented by variegated and lethal forms. It was noted that with the prolongation of temperature treatment the frequency of occurrence of chlorophyll abnormalities in M_1 and the mutation frequency in M_2 decreased.

To summarize these results, we conclude that the plastomic mutant en-chlorina-5 displays a higher tolerance to the action of HS as compared to other sunflower lines. This plastomic mutant is characterized by an alteration in the fatty-acid composition of lipids, activity of antioxidant enzymes, and in the level of products of peroxide oxidation of lipids both in the root system and in leaf tissues [18, 29]. This serves as a basis for increasing the cell cycle stability in apical root cells and resistance of cotyledonous epidremal cells to HS, as well as for increasing stability of enzymatic complexes that form the photosynthetic apparatus of plant cells.

It is possible that the occurrence of a mutation in chloroplast DNA increases heterogeneity of the cell genetic system, which serves as a basis for enhancing the adaptive potentialities due to selection of DNA-containing cytoplasmic organelles able not only to function effectively under conditions deviating from normal, but also to maintain the vital activity of the whole complex hierarchic system of a plant cell.

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