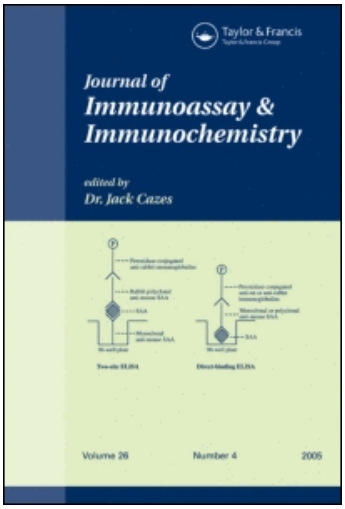

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STRESS PROTEIN CSP 310 CAUSES OXIDATION AND PHOSPHORYLATION UNCOUPLING DURING LOW-TEMPERATURE STRESS ONLY IN CEREAL BUT NOT IN DYCOTYLEDON MITOCHONDRIA

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ABSTRACT

It was found that an addition of antiserum obtained against stress protein 310 kD increased coupling of oxidation and phosphorylation in mitochondria isolated from cold-stress winter rye shoots and had no influence on dycotiledon mitochondria (pumpkins and sunflower). The data obtained showed a difference between molecular weights of dycotyledon polypeptides with immunochemical affinity to CSP 310 and CSP 310 subunits. It was shown that low-temperature

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stress caused a transition to a low-energy state (''cold uncoupling'') of free from endogenous free fatty acid cereal mitochondria. At the same time, this ''cold uncoupling'' in mitochondria of dycotyledon species investigated was not detected. We suppose that a special mechanism of low-temperature stress reaction in mitochondria dealing with uncoupling activity of stress protein CSP 310 exists in cereals.

INTRODUCTION

It is known that low-temperature stress causes significant changes in energetic metabolism of different organisms. It was shown that a short-term cold exposure of cold-adapted pigeons resulted in significant decrease of P/O ratio in muscle mitochondria(1). This reaction to cold stress, called ''thermoregulatory uncoupling'' or ''cold uncoupling'' was later shown in brown fat mitochondria of mice and other animals.(2) Now it is considered that the major consequence of the uncoupling of respiration is the activation of substrate oxidation and dissipation of energy obtained as heat. In addition to adaptive thermogenesis participation, it was proposed that cold uncoupling prevents the accumulation of oxygen radicals generated by mitochondria and could be involved in regulation of redox conditions and metabolic pathways.(3)

At the same time, uncoupling of oxidation and phosphorylation occurring in cold-resistant winter wheat mitochondria during low-temperature stress was observed.(4) In that study, it was shown that chilling of winter wheat seedlings for 1 h from 20 to −4°C caused an increase in non-phosphorylative respiration and a decrease in respiratory control. Currently, some plant uncoupling proteins – PUMP,(5) AtPUMP,(6) and StUCP(7) are known. It was supposed that these proteins can regulate the mitochondrial energetic functions during low-temperature stress.(3,7) These plant uncoupling proteins are homologues of the well-known mammalian and human uncoupling protein UCP-1 and function by a ''fatty acid cycling'' mechanism as mammalian uncoupling proteins.(8–9) Their activity *in vitro* depends upon the presence of free fatty acids in mitochondria incubation medium.(10) At the same time, some data show that infiltration of winter wheat seedlings by procaine, which inhibits phospholipase A_2, did not fully inhibit thermogenesis in mitochondria of the cold-resistant winter wheat cultivars.(4) Recently, the native stress protein with molecular weight 310 kD (CSP 310), from cold-stressed winter rye and winter wheat seedlings, was isolated(11) and it was shown that this protein has an uncoupling activity.(12)

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CSP 310 was found in cytoplasmatic(13) and in mitochondrial(14) proteins of different cereals – winter wheat, winter rye, and maize. It was found that this protein is constituently synthesized by 3 day-old cereal shoots(11) but did not cause significant uncoupling. The constituently synthesized form of CSP 310 changed its features and converse in the form with high uncoupling activity during cold stress.(14–15) In vivo amount of this protein in mitochondria of cereals increased under these conditions.(14) Similar data were obtained during the study of CSP 310 association with mitochondria during modeling cold stress in vitro.(16) Previously, it was shown that antiserum against CSP 310 caused coupling of oxidation and phosphorylation in the mitochondria isolated from non-stressed cereals with different cold resistance, but had no influence on pea mitochondria.(17) At the same time, the effect of anti-CSP 310 antiserum on mitochondria isolated from stressed cereals and dycotyledons, and the presence in dycotyledon polypeptide spectra immunochemically related to CSP 310 subunits polypeptides were not studied.

So, the aims of this study are

1. to determine an influence of anti-CSP 310 antiserum on the oxidation and phosphorylation coupling in cereal and dycotyledon mitochondria isolated from cold-stressed shoots,
2. to compare the spectra of immunochemically related to CSP 310 subunits polypeptides in dycotyledon and cereals, and
3. to study the influence of the cold stress on cereal and dycotyledon mitochondria activity.

EXPERIMENTAL

Three day-old etiolated shoots of winter wheat (Triticum aestivum L), winter rye (Secale cereale L.), and maize (Zea mays L.) and six day-old etiolated shoots of pea (Pisum sativum L.), pumpkins (Cucurbita pepo L.), and sunflower (Helianthus sativum L.) germinated on moist paper at 26°C were used in this work.

The duration of low-temperature stress was 1 h for all species investigated. Stress intensity was determined experimentally and was −2°C for the most cold-resistant winter rye, − 1°C for winter wheat, pea, and sunflower and 0°C for maize and pumpkins.

Mitochondria were extracted from seedling shoots by differential centrifugation, as described previously.(18) The mitochondria isolated were resuspended in the following medium: 40 mM MOPS-KOH buffer (pH 7.4), 300 mM sucrose, 10 mM KCl, 5 mM EDTA, 1 mM $MgCl_2$, 4 mM ATP,

6 mM ADP, 10 mM malate, and 10 mM glutamate. To eliminate the free fatty acids uncoupling effect, 0.1% bovine serum albumin was added to the mitochondria isolation medium.

The activity of mitochondria was recorded polarographically at 27°C using a platinum electrode of a closed type in a 1.4 mL volume cell.(19) The reaction mixture contained 125 mM KCl, 18 mM KH_2PO_4, 1 mM $MgCl_2$ and 5 mM EDTA, pH 7.4. 10 mM Malate, in the presence of 10 mM glutamate, was used as an oxidation substrate.

The isolation and purification of winter wheat CSP 310 and obtaining of anti-CSP 310 antiserum were performed in accordance with the previously described method.(11) Non-immune antiserum was obtained from non-immunised rabbits. Antiserums were dissolved in the incubation medium and were added in the polarographic cell to incubated in state 4 mitochondria in the first cycle of phosphorylation. The concentration of anti-CSP 310 antiserum was from 1.2 to 3.6 mg per 1 mL of incubation medium. The concentration of non-immune antiserum was 1.2 mg per 1 mL of incubation medium.

The concentrations of mitochondrial protein and anti-CSP 310 antiserum were analysed by Lowry method.(20) Polarograms were used to calculate the rates of phosphorylative respiration (state 3), non-phosphorylative respiration (state 4), respiratory control by Chance-Williams and the ADP:O ratio.(19)

SDS-electrophoresis was performed in LaemmLi system.(21) After electrophoresis, proteins were stained by Coomassie R-250. The relative molecular weights of the proteins were determined by using an LMW kit of markers (Sigma, USA). Western-blotting was performed as described previously.(13)

All experiments were performed in three biological replications. The data obtained were analysed statistically, i.e., arithmetic means and S.D. ($P > 0.95$) were determined.

RESULTS AND DISCUSSION

Previously, we showed that an addition of anti-CSP 310 antiserum, obtained against cold stress protein CSP 310, to cereal mitochondria isolated from non-stressed seedlings caused an increase of the oxidation and phosphorylation coupling. At the same time, an addition of anti-CSP 310 antiserum did not have an influence on pea mitochondrial activity.(17) Whereas the content of CSP 310 in cells of different cereals was increased during cold stress,(11) we made an attempt to determine an effect of anti-CSP 310 antiserum on energetic activity and degree of oxidation and

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phosphorylation coupling of mitochondria isolated from cold-stressed cereal and dycotyledon seedlings. Because the CSP 310 content in winter rye cells increased more significantly than in other cereals investigated during cold stress, mitochondria isolated from stressed winter rye shoots were used for this study.(13) For comparison, mitochondria isolated from cold-stressed dycotyledon (pumpkins and sunflower) seedlings were used.

An addition of anti-CSP 310 antiserum to incubated at state 4 winter rye mitochondria isolated from stressed seedlings (−1°C, 1 h) significantly decreased the non-phosphorylative respiration rate and increased the respiratory control coefficient (Fig. 1). These changes witness the increase of oxidation and phosphorylation coupling in mitochondria. It is necessary to note that an addition of the non-immune antiserum did not cause this effect (Fig. 1). An increase of the added antiserum in amounts up to 1.8 mg per 1 mg of mitochondrial protein caused the increase of coupling up to 3.5 times. Subsequent increase of the added antiserum content had no influence on the coupling effect (Fig. 1). As to the influence of anti-CSP 310 antiserum on winter rye mitochondria, isolated from stressed at −2°C shoots, we found that it was necessary to add greater amounts of antiserum to obtain the same effect as on stressed at −1°C winter rye shoots. For example, to obtain an increase of coupling up to 3.8 times, it was necessary to add 2.4 mg of antiserum (Fig. 2). We suppose that this fact depends on the greater amounts associated with mitochondria CSP 310 in stressed at −2 °C winter rye shoots.

Therefore, an addition of anti-CSP 310 antiserum to incubated at state 4 winter rye mitochondria caused an increase of the oxidation and phosphorylation coupling. We suppose that this coupling effect takes place because of precipitation of associated with mitochondria stress protein CSP 310, that increased in amounts during low-temperature stress.(14)

An addition of anti-CSP 310 antiserum to mitochondria isolated from stressed dycotyledon seedlings did not cause any significant increase of respiratory control coefficient and oxidation and phosphorylation coupling. Mitochondrial energetic activity of stressed pumpkins and sunflower did not change after addition of anti-CSP 310 antiserum (Table 1).

Thus, anti-CSP 310 antiserum caused the increase of oxidation and phosphorylation coupling in the cereal mitochondria isolated from chilled shoots that possibly deal with precipitation of the endogenous CSP 310 and do not cause this effect in the dycotyledon mitochondria. We supposed that non-affectivity of the anti-CSP 310 antiserum on the dycotyledon mitochondria deals with the absence the native CSP 310 in spectra protein of these plants.

As was previously shown, in cereals all native CSP 310-like proteins consist of various combinations of two types of subunits with molecular

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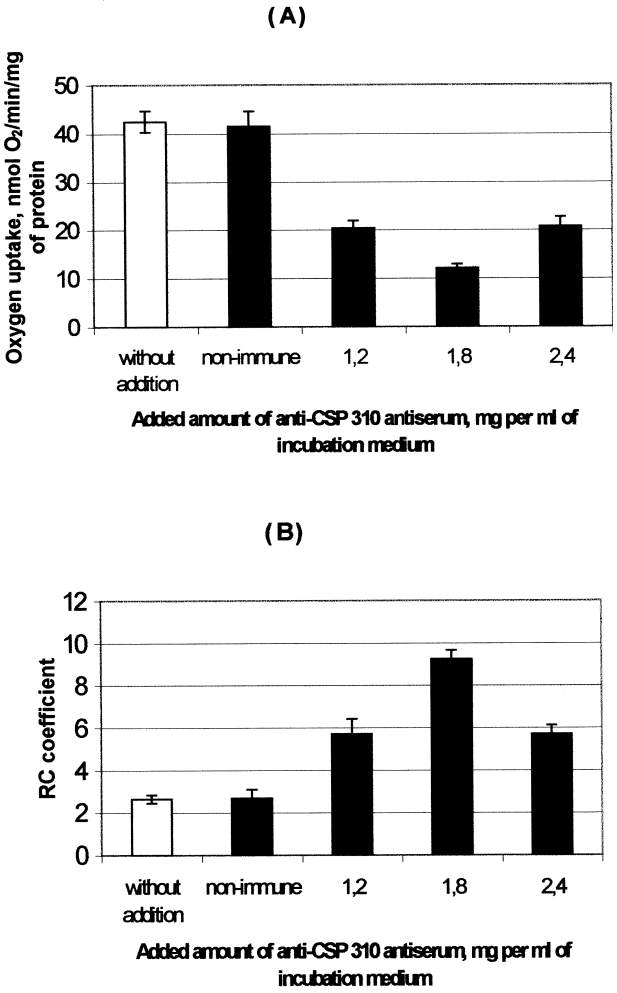

Figure 1. An influence of addition of anti-CSP 310 antiserum to incubated at state 4 isolated from stressed at -1°C winter rye shoots mitochondria on the rate of state 4 respiration (A) and respiratory control coefficient (B).

weights 56 and 66 kD.(13) At the same time, SDS-electrophoresis followed by western-blotting with anti-CSP 310 polyclonal antibodies showed the presence of polypeptides, immunochemically related to CSP 310 subunits, with other molecular weights in dycotyledon polypeptide spectra (Fig. 3). In its spectrum, CSP 310 subunits with molecular weights 56 and 66 kD were not detected, but a number of proteins with other molecular weights were

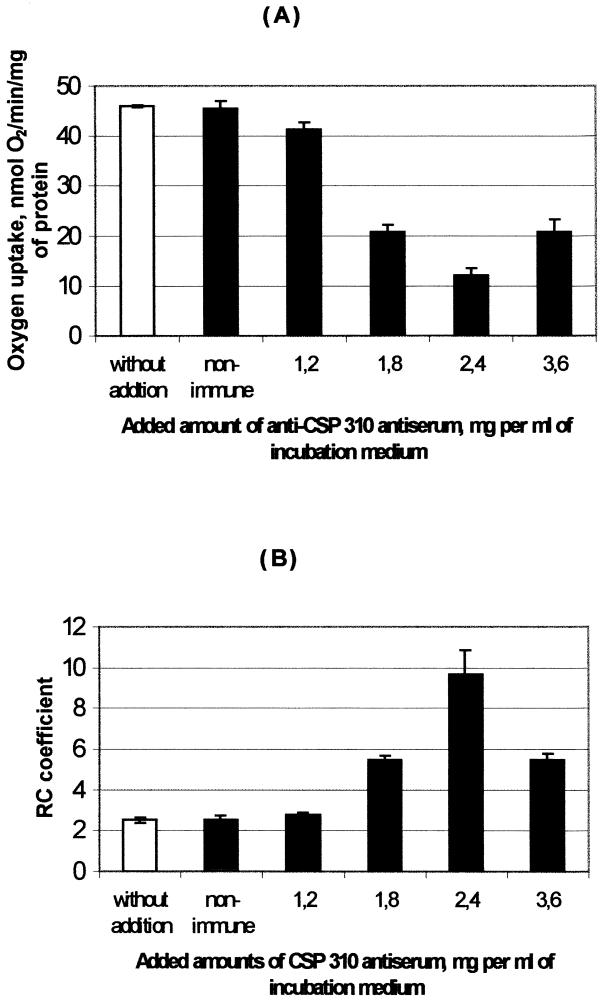

Figure 2. An influence of addition of anti-CSP 310 antiserum to incubated at state 4 isolated from stressed at −2°C winter rye shoots mitochondria on the rate of state 4 respiration (A) and respiratory control coefficient (B).

found. Thus, there were polypeptides with molecular weights 100, 85, 70, 59, 41, 39, 38, and 35 kD in pea, 80, 75, 70, 59, and 50 kD in pumpkins and 85, 70, 59, and 50 kD in sunflower (Fig. 3).

The data obtained showed an appearance of 100 kD polypeptide and increasing of 59 kD polypeptide amounts in the stressed pea seedling protein spectrum (Fig. 3). In stressed pumpkins seedling spectrum, increased

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Table 1. The Influence of Anti-CSP 310 Antiserum on the Rate of Non-phosphorylative (State 4) Respiration and Respiratory Control Coefficient (RC) of Mitochondria Isolated from Different Plant Species. 10 mM Malate in the Presence of 10 mM Glutamate Was Used as an Oxidation Substrate. $M \pm SD$, $n = 3$

Plant Species	Before Addition of Anti-CSP 310 Antiserum		After Addition of 1.2 mg of Anti-CSP 310 Antiserum per 1 mL of Incubation Media	
	State 4*	RC	State 4*	RC
Pumpkins	17.3 ± 1.4	4.71 ± 0.15	16.1 ± 0.8	5.03 ± 0.24
Sunflower	22.8 ± 1.6	3.46 ± 0.34	22.5 ± 1.1	3.50 ± 0.17

*Rate of oxygen uptake (nmol O_2/min/mg protein).

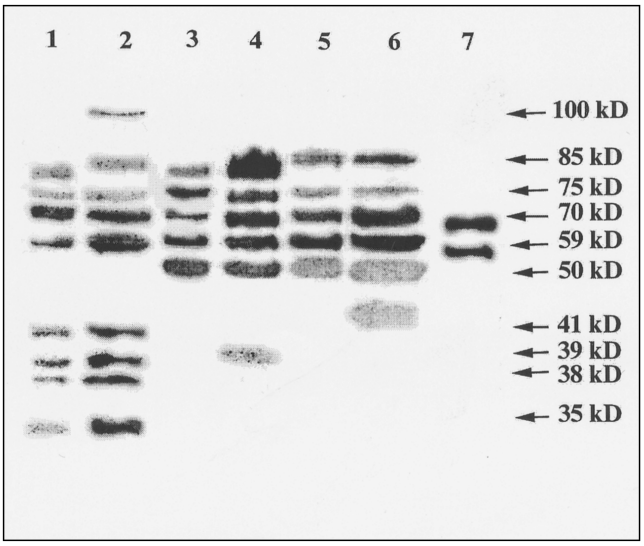

Figure 3. Western-blotting of control (1) and stressed at $-1^{\circ}C$ (2) pea, control (3) and stressed at $0^{\circ}C$ (4) pumpkins, control (5) and stressed at $-1^{\circ}C$ (6) sunflower seedlings and (7) winter rye CSP 310 with polyclonal antibodies to CSP 310.

amounts of 85, 70, and 59 kD polypeptide (especially 85 kD polypeptides) were detected. In stressed sunflower seedlings, an increase of 85 and 59 kD polypeptides was detected (Fig. 3). The absence of mitochondrial activity changes in these plant species' stressed seedlings under the influence of anti-CSP 310 antiserum allows us to conclude that these cold-stress related

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polypeptides carry out different functions in dycotyledons than 56 and 66 kD subunits of CSP 310 in cereals.

The difference in polypeptide spectra of the dycotyledon proteins immunochemically related to CSP 310 allows us to propose that the reaction of dycotiledon mitochondria on cold stress can be different from that of cereal mitochondria. To verify this suggestion, we studied the influence of low-temperature stress on respiratory activity of cereal and dycotiledon mitochondria.

For freshly isolated non-stressed shoots of all species investigated, mitochondria had a high energetic activity and a high degree of coupling of oxidation and phosphorylation (Tables 2, 3). The value of the respiratory control coefficient was from 3.34 in winter rye to 4.57 in pumpkins. ADP:O ratio was high as well.

Cold stress caused significant changes in energetic activity of mitochondria isolated from stressed cereal shoots (Table 2). These changes were observed in all the cereal species investigated, including heat-loving maize. In cereal mitochondria, a slight increase in phosphorylative (state 3) respiration, significant increase in non-phosphorylative (state 4) respiration, and a decrease in respiratory control coefficient and ADP:O ratio were detected. So, mitochondria of all cereal species investigated, including heat-loving maize, are transformed to a low-energy state during low-temperature stress. The greatest decrease of oxidation and phosphorylation coupling in mitochondria from stressed winter rye and winter wheat shoots was observed.

Table 2. Energetic Activity of the Mitochondria from Control and Stressed Seedling Shoots of Some Cereal Species. 10 mM Malate in the Presence of 10 mM Glutamate Was Used as an Oxidation Substrate. $M \pm SD$, $n = 6$

Variant	Rate of Oxygen Uptake nmol O_2/min/mg Protein		RC	ADP:O Ratio
	State 3	State 4		
Winter rye				
Control	93.7 ± 7.9	27.9 ± 2.7	3.34 ± 0.04	2.97 ± 0.01
−2°C, 1 h	110.5 ± 6.6	44.8 ± 2.8	2.47 ± 0.03	2.17 ± 0.10
Winter wheat				
Control	98.0 ± 4.3	24.8 ± 1.7	3.97 ± 0.09	2.90 ± 0.13
−1°C, 1 h	$106.8 \pm 6{,}0$	41.7 ± 1.4	2.56 ± 0.09	2.82 ± 0.04
Maize				
Control	104.5 ± 4.4	27.8 ± 1.5	3.76 ± 0.12	2.71 ± 0.05
0°C, 1 h	111.8 ± 4.9	33.5 ± 1.6	3.35 ± 0.02	2.46 ± 0.07

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This decrease of coupling occurred because of an increase of state 4 respiration rate (Table 2). In winter rye mitochondria, isolated from stressed seedlings, an increase in state 4 respiration was about 60.7% to control and decrease in respiratory control was about 24%. Decrease of ADP:O ratio in winter rye mitochondria isolated from stressed winter rye seedlings was about 27% from control. Changes in winter wheat mitochondria were significant too. In this case, the increase in non-phosphorylative respiration was 68.5% and decrease in respiratory control coefficient was 35.5% of control (Table 2). At the same time, ADP:O ratio in stressed winter wheat mitochondria decreased only about 3%.

Mitochondria isolated from heat-loving maize also showed the same changes in their energetic activity during low-temperature stress, but at a lesser degree. The increase in non-phosphorylative respiration in stressed maize mitochondria was about 20% and decrease in respiratory control coefficient was about 11% (Table 2).

On the other hand, the study of the influence of low-temperature stress on energetic activity of dycotyledon mitochondria showed the absence of changes in coupling parameters as a result of low-temperature stress in mitochondria that were free from endogenous free fatty acid (Table 3). All values of energetic activity of stressed pea, pumpkins, and sunflower seedling mitochondria did not differ from values of control variant mitochondria. Low temperature stress did not cause in all dycotyledon species investigated, including rather cold-resistant pea, neither an increase in non-phosphorylative respiration nor a decrease in respiratory control

Table 3. Energetic Activity of the Mitochondria from Control and Stressed Seedling Shoots of Some Dycotyledon Species. 10 mM Malate in the Presence of 10 mM Glutamate Was Used as an Oxidation Substrate. $M \pm SD$, $n = 6$

	Rate of Oxygen Uptake nmol O_2/min/mg Protein			
Variant	State 3	State 4	RC	ADP:O Ratio
Pumpkins				
Control	82.4 ± 3.4	17.8 ± 1.1	4.57 ± 0.29	3.03 ± 0.05
0°C, 1 h	81.6 ± 1.4	17.3 ± 0.6	4.73 ± 0.12	2.82 ± 0.04
Pea				
Control	82.1 ± 4.5	20.1 ± 1.1	4.08 ± 0.01	2.59 ± 0.15
−1°C, 1 h	80.6 ± 1.4	19.9 ± 1.1	4.05 ± 0.07	2.57 ± 0.14
Sunflower				
Control	75.7 ± 6.1	23.4 ± 2.6	3.33 ± 0.08	2.41 ± 0.15
−1°C, 1 h	76.0 ± 7.0	22.6 ± 1.5	3.36 ± 0.30	2.39 ± 0.08

coefficient. ADP:O ratio did not change as a result of low-temperature stress, either. In connection with this fact, we can suppose that resistance of pea seedlings to low-temperature is provided by mechanisms different from those in cereals.

The degree of transition of cereal mitochondria to a low-energy state is well correlated with the amounts of stress uncoupling protein CSP 310 in cytoplasmatic proteins of species investigated. Winter rye and winter wheat mitochondria showed significant changes in their energetic activities because of low-temperature stress. At the same time, the highest concentration of CSP 310 was found(13) in cytoplasmatic proteins of these species. Changes in maize mitochondrial activity are significantly less, and CSP 310 concentration in its cytoplasmatic proteins is lower.(13)

On the basis of data obtained for spectra of immunochemically related CSP 310 subunit polypeptides in dycotyledons and the absence of changes in mitochondrial activity in stressed seedlings of these species, we can suppose that uncoupling of oxidation and phosphorylation in cereal mitochondria during cold stress is connected with non-free fatty acid uncoupling which depends on the mechanism deal with CSP 310 activity.

Based on results obtained, we can conclude that, in cereals during low-temperature stress, the transition of mitochondria to a low-energy state occurs because of CSP 310 uncoupling activity. The degree of this effect depends on the intensity of cold stress.

Therefore, our results show that the special mechanism of mitochondrial reaction to low-temperature stress deals with uncoupling activity of stress protein CSP 310 which exists in cereals; it can play the important role in plants' adaptation to cold stress. At the same time, this mechanism was not found in dycotyledons, even in the cold-tolerant pea. We can suppose that resistance of dycotyledon species to low-temperature stress is provided by mechanisms that differ from those for cereals.

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