BMC Plant Biology

Research article

Influence of CSP 310 and CSP 310-like proteins from cereals on mitochondrial energetic activity and lipid peroxidation *in vitro* and *in vivo*

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Published: 26 September 2001

BMC Plant Biology 2001, **1**:1

Received: 25 June 2001
Accepted: 26 September 2001

This article is available from: http://www.biomedcentral.com/1471-2229/1/1

Abstract

Background: The development of chilling and freezing injury symptoms in plants is known to frequently coincide with peroxidation of free fatty acids. Mitochondria are one of the major sources of reactive oxygen species during cold stress. Recently it has been suggested that uncoupling of oxidation and phosphorylation in mitochondria during oxidative stress can decrease ROS formation by mitochondrial respiratory chain generation. At the same time, it is known that plant uncoupling mitochondrial protein (PUMP) and other UCP-like proteins are not the only uncoupling system in plant mitochondria. All plants have cyanide-resistant oxidase (AOX) whose activation causes an uncoupling of respiration and oxidative phosphorylation. Recently it has been found that in cereals, cold stress protein CSP 310 exists, and that this causes uncoupling of oxidation and phosphorylation in mitochondria.

Results: We studied the effects of CSP 310-like native cytoplasmic proteins from a number of cereal species (winter rye, winter wheat, *Elymus* and maize) on the energetic activity of winter wheat mitochondria. This showed that only CSP 310 (cold shock protein with molecular weight 310 kD) caused a significant increase of non-phosphorylative respiration. CSP 310-like proteins of other cereals studied did not have any significant influence on mitochondrial energetic activity. It was found that among CSP 310-like proteins only CSP 310 had prooxidant activity. At the same time, *Elymus* CSP 310-like proteins have antioxidant activity. The study of an influence of infiltration by different plant uncoupling system activators (pyruvate, which activates AOX, and linoleic acid which is a substrate and activator for PUMP and CSP 310) showed that all of these decreased lipid peroxidation during cold stress.

Conclusions: Different influence of CSP 310-like proteins on mitochondrial energetic activity and lipid peroxidation presumably depend on the various subunit combinations in their composition. All the plant cell systems that caused an uncoupling of oxidation and phosphorylation in plant mitochondria can participate in plant defence from oxidative damage during cold stress.

Background

The development of chilling and freezing injury symptoms in plants is known to frequently coincide with peroxidation of free fatty acids [1]. Peroxide and malondialdehyde levels are often increased by freezing and thawing stress suggesting peroxidation of lipids [2]

that result in structural and functional membrane and membrane protein changes [3]. It has been shown that mitochondria are one of the major sources of superoxide, which is a powerful oxidant radical, in chilling-sensitive plant tissues at low temperatures [4]. Even in non-stress conditions, 1–2 % of oxygen reduced in mitochondria by iron-sulfur centers in complex I and partially by reduced ubiquinone and cytochrome *b* in complex III is constitutively converted to superoxide [5,6]. Mitochondria are also a major site for the accumulation of low molecular weight Fe^{2+} complexes, which induce lipid peroxidation in membranes [7,8].

It has also been shown that cold stress causes an increase in antioxidant levels in many plant species. Furthermore, it has been found that different isoenzymes of glutathione reductase are expressed during cold acclimation of red spruce (*Picea rubens*) needles. One of them has been proposed to be a cold-acclimation protein [9]. Recently it was suggested that in mitochondria there was another physiological mechanism involved into antioxidant defense system of cell. V.P. Skulachev supposed that uncoupling of oxidation and phosphorylation in mitochondria during oxidative stress could decrease ROS generation by mitochondrial respiratory chain [10]. This point of view was supported by recent studies of plant UCP-like uncoupling proteins such as PUMP and others. For example, it was found that the inhibition of PUMP activity in isolated potato tuber mitochondria significantly increased mitochondrial H_2O_2 generation. It was also found that such substrates of UCP-like uncoupling proteins as linoleic acid and other free fatty acids reduced mitochondrial H_2O_2 generation [11,12]. However, it is necessary to note that reactions of isolated organelles and whole plant cell on the same treatment can differ. At the same time, it is known that PUMP and other UCP-like proteins are not the only uncoupling system in plant mitochondria. All the plants have such a mechanism in mitochondria as alternative cyanide-resistant oxidase (AOX) whose activation during cold stress causes uncoupling of respiration and oxidative phosphorylation [13]. Recently it has been found that in cereals, such as winter wheat and winter rye, cold stress protein CSP 310 is present [14] and also causes uncoupling of respiration and oxidative phosphorylation in mitochondria during cold stress [15]. It was found that an addition of CSP 310 to isolated winter wheat mitochondria induced ascorbat-dependent and NADH-dependent lipid peroxidation systems unlike other known uncoupling proteins [16]. On the other hand, it has been shown that inhibition of CSP 310 by specific antiserum increases lipid peroxidation in isolated mitochondria [17]. Therefore, it is interesting to determine what CSP 310 activity, prooxidant or antioxidant is the most pronounced in whole winter wheat shoots during cold stress.

Meanwhile, the presence of a "family" of proteins immunochemically related to CSP 310 in cereals has been established. Earlier it has been shown that CSP 310-like native proteins are present in cytoplasm [18] and mitochondria [19] of cereals with different cold tolerance, and that CSP 310 could associate with and dissociate from mitochondria [20]. At the same time, CSP 310 was found in mitochondrial proteins of all species investigated, but this protein was found in winter rye and in lower amounts in winter wheat cytoplasmic proteins only. By using affine chromatography on a column with immobilized on BrCN-activated Sepharose anti-CSP 310 antiserum preparations of CSP 310-like cytoplasmic proteins from winter rye, winter wheat, maize and *Elymus* were obtained. These preparations consisted of 470 KD, 310 kD, 230 kD, about 140 kD, 66 kD and 56 kD proteins from winter rye, 310 kD, 230 kD, about 140 kD, 66 kD and 56 kD from winter wheat, 230 kD, about 140 kD, 66 kD and 56 kD from maize and 380 – 330 kD, 230 kD, about 140 kD, 66 kD and 54 kD proteins from *Elymus*. All these proteins consist of two types of subunits [18]. At the same time, this difference in spectra of native cereal proteins allows us to suppose a difference between their influence on mitochondrial energetic activity and lipid peroxidation.

The aim of this work is to examine the influence of proteins related to CSP 310 from some cereal species on energetic activity and lipid peroxidation in winter wheat mitochondria *in vitro* we also aim to examine the influence of activation of plant uncoupling systems on lipid peroxidation in winter wheat seedlings shoots during cold stress.

Results and Discussion

An influence of proteins immunochemically related to CSP 310 from winter rye, winter wheat, *Elymus* and maize on functional activity of winter wheat mitochondria during hypothermia *in vitro* is shown in Table 1. At the beginning of incubation freshly isolated from winter wheat seedlings shoots mitochondria had a high energetic activity and a high degree of oxidation and phosphorylation coupling. An incubation of these mitochondria for 30 min at 0°C with immunochemically related to CSP 310 proteins from winter rye caused about 35 % increase of non-phosphorylative (state 4) respiration and decrease of respiratory control and ADP:O ratio also (Table 1). At the same time, an addition to incubated mitochondria immunochemically related to CSP 310 proteins from *Elymus* and winter wheat failed to result in any significant changes in their activity. As to proteins from CSP 310 "family" from maize, they caused only non-significant increasing of non-phosphorylative (state 4) respiration (Table 1). It is necessary to note that at this study we used mitochondria purified from free fatty acids by BSA

Table 1: An influence of winter rye, winter wheat, *Elymus* and maize proteins, immunochemically related to CSP 310, on the oxidative activity of winter wheat mitochondria.

Incubation, min	Rate of oxygen uptake nmol O_2/min/mg protein		Respiratory control coefficient	ADP:O ratio
	State 3	State 4 Control		
0	78.8 ± 5.4	25.0 ± 2.0	3.15 ± 0.12	2.57 ± 0.11
30	77.8 ± 7.1	25.5 ± 0.2	3.04 ± 0.17	2.20 ± 0.24
With addition of winter rye CSP 310-like proteins				
0	81.0 ± 4.6	26.8 ± 1.0	3.02 ± 0.11	2.68 ± 0.06
30	79.7 ± 7.8	36.1 ± 1.8	2.30 ± 0.24	2.00 ± 0.08
With addition of winter wheat CSP 310-like proteins				
0	77.5 ± 5.4	25.7 ± 1.4	3.01 ± 0.14	2.56 ± 0.08
30	76.4 ± 7.8	26.3 ± 1.4	2.90 ± 0.37	2.06 ± 0.13
With addition of maize CSP 310-like proteins				
0	80.1 ± 2.7	26.7 ± 1.7	3.00 ± 0.22	2.48 ± 0.12
30	76.8 ± 2.3	29.4 ± 1.0	2.60 ± 0.08	1.91 ± 0.10
With addition of *Elymus* CSP 310-like proteins				
0	74.5 ± 4.6	24.5 ± 1.4	3.04 ± 0.14	2.56 ± 0.16
30	73.4+7.3	25.0 ± 1.1	2.90 ± 0.07	2.26 ± 0.10

addition so this uncoupling was caused by an addition of proteins but not an increase of endogenous free fatty acids content. The difference between used preparations of CSP 310-like proteins from cereals is only in its subunit composition. Therefore, based on the data we could conclude that only CSP 310 but not any other composition of its subunits caused an uncoupling of oxidation and phosphorylation in mitochondria.

The study of the influence of CSP 310-like cytoplasmic proteins from winter rye, winter wheat, maize and *Elymus* on lipid peroxidation shows that immunochemically related to CSP 310 proteins from winter wheat, maize and *Elymus* did not have such prooxidant effect as CSP 310-like proteins from winter rye (Fig. 1). At the same time if proteins from winter wheat and maize did not have significantly influence on lipid peroxidation, proteins from *Elymus* do have an antioxidant activity in all systems studied (Fig. 1). In particular, proteins from CSP 310 "family" from *Elymus* inhibit NADH-dependent lipid peroxidation more than two times as compared to the variant without addition of any proteins.

All the proteins of CSP 310 "family" were previously shown to consist of two types of subunits [18]. The difference between these proteins is only in different composition of two types of subunits in native proteins. In accordance with this fact, the difference between their influence on energetic activity and lipid peroxidation can deal with the difference between their native structures. Therefore, we suppose that non-active low molecular weight combinations of CSP 310 subunits can be a "de-

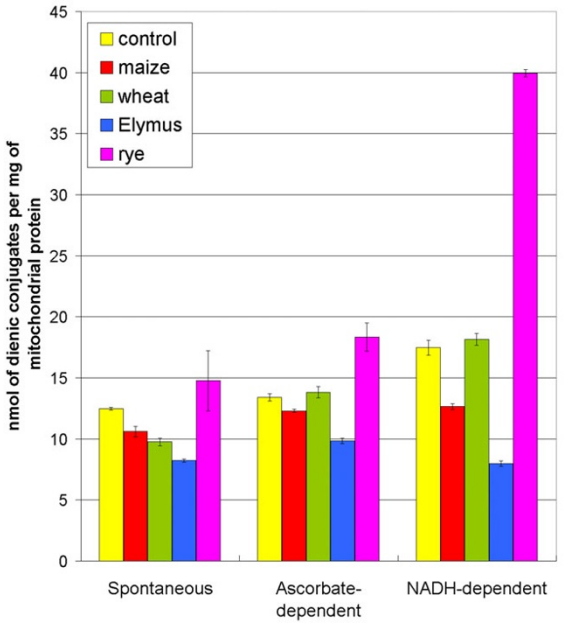

Figure 1
An influence of winter rye, winter wheat, *Elymus* and maize proteins, immunochemically related to CSP 310, on lipid peroxidation in winter wheat mitochondria at 37°C (nMol of dienic conjugates per mg of mitochondrial protein). M ± SD, *n* = 6.

pot" that allows to rapidly increase CSP 310 concentration during cold stress. Because the reaction of isolated mitochondria and of the whole plant on CSP 310 treatment can differ, we studied an influence of CSP 310 infiltration of winter wheat seedlings shoots on lipid peroxidation and oxygen consumption.

The results obtained showed that cold stress treatment caused about 20 % increasing of dienic conjugates formation in winter wheat shoots tissues (Fig. 2). These data are well correlated with data obtained by many investigators on the influence of cold stress on lipid peroxidation in plant tissues [1,2]. At the same, time we observed a slightly increase of oxygen consumption of winter wheat shoots during cold stress (Fig. 3).

An infiltration of winter wheat shoots by all plant uncoupling mitochondrial systems activators investigated (pyruvate, that activate AOX [13], linoleic acid, which is a substrate and activator of PUMP [12] and CSP 310) reduced the rate of lipid peroxidation during cold stress (Fig. 2). It is interesting to note, that all of them caused about 30 % decrease of dienic conjugates formation, and these values were slightly lower but were not statistically different from the value of dienic conjugate contents in non-stressed winter wheat shoots (Fig. 2). The study of an influence of these activators on oxygen consumption in winter wheat shoots during cold stress showed that all of them caused an increase of oxygen consumption values (Fig. 3). It is necessary to note, that, if the infiltration of winter wheat shoots by pyruvate and CSP 310 caused about 20–25 % increasing of oxygen consumption, their infiltration by linoleic acid caused about 100 % increasing of oxygen consumption. These data allow us to suppose that infiltration of winter wheat shoots by linoleic acid can strongly activate UCP-like plant uncoupling proteins and other members of the mitochondrial carrier protein family. On the other hand, this effect can deal not only with PUMP activation but also with the direct uncoupling effect of free fatty acids that are powerful uncouplers by themselves. Similarly, the influence of pyruvate infiltration on lipid peroxidation may be caused by its own antioxidant effect, but at the same time its effect on oxygen consumption during cold stress allows us to suppose that it can deal with its influence on mitochondrial energetic activity too.

Conclusions

So, based on the data obtained we can conclude that during cold stress all the studied winter wheat uncoupling systems can be involved in plant defense against oxidative stress. Low-molecular weight CSP 310-like proteins obtained from cereals investigated decreased lipid peroxidation in mitochondria but did not influence the energetic activity of plant mitochondria. Only CSP 310 increased lipid peroxidation in mitochondria damaged by inducing ascorbat-dependent and NADH-dependent lipid peroxidation systems.

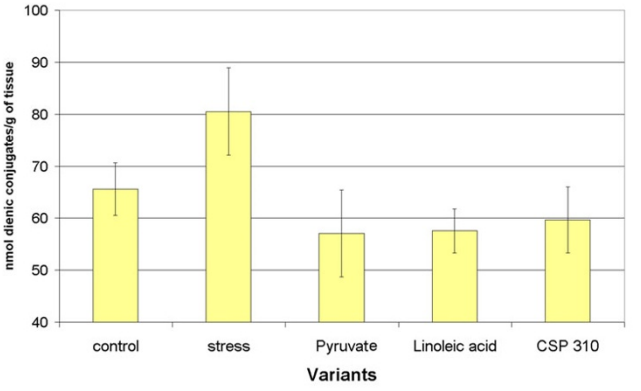

Figure 2
An influence of activators of known plant uncoupling proteins on lipid peroxidation in winter wheat shoots during cold stress. M ± SD, *n* = 6.

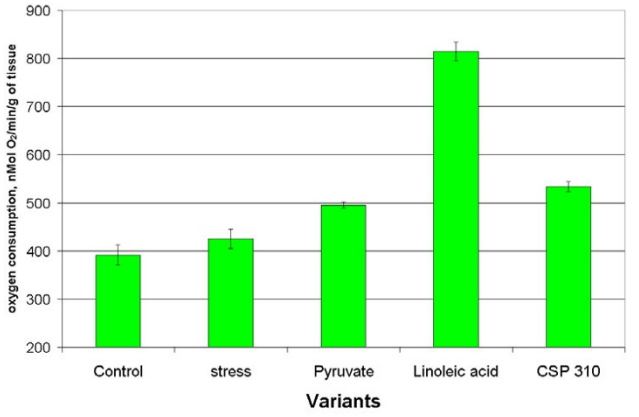

Figure 3
An influence of activators of known plant uncoupling proteins on oxygen consumption of winter wheat shoots during cold stress. M ± SD, *n* = 6.

Materials and Methods

Three day old etiolated shoots of winter wheat (*Triticum aestivum* L., cv. Irkutskaya Ozimaya) grown on moist paper at 26°C, were used in this work.

Crude and purified mitochondria were isolated from winter wheat shoots by the method described previously using discontinuous Percoll gradient consisting of 18%, 23%, 40% Percoll [15,21]. Purity and integrity of mitochondria were determined by measurement of cytochrome *c* oxidase activity (EC 1.9.3.1) [22]. The mitochondrial proteins were measured by the Lowry method [23].

The isolated mitochondria were resuspended in the following medium: 20 mM MOPS-KOH buffer (pH 7.4), 300 mM sucrose, 10 mM KC1, 5 mM EDTA, 1 mM $MgCl_2$, 4 mM ATP, 4 mM ADP, 10 mM malate and 10 mM glutamate. In these experiments 0.5 mg of immunochemically related to CSP 310 proteins were added to mitochondrial suspension per 1 mg of mitochondrial protein. Energetic activity of mitochondria was analyzed immediately after isolation and after 30 min of incubation at 0°C. The aliquots of mitochondrial suspension were taken and the mitochondrial energetic activity was recorded polarographically at 27°C. The reaction medium 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. Polarogrammes 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 [24].

Proteins, immunochemically related to winter rye stress protein CSP 310 from winter rye, winter wheat, *Elymus* and maize were isolated as described previously using affine chromatography on a column with BrCN-activated Sepharose with immobilized anti-CSP 310 antiserum [18].

The rate of lipid peroxidation was determined by measurement of the primary products of lipid peroxidation – conjugated diene formation. Mitochondria were incubated in the incubation media contained 175 mM KCl and 25 mM Tris-HCl (pH 7.4). For induction of the ascorbate – dependent lipid peroxidation system 1 mM ascorbate, 1 mM ADP and 20 mkM Fe^{2+} were added to the incubation media. For induction of the NADH – dependent lipid peroxidation system 1 mM NADH, 1 mM ADP and 20 mkM Fe^{2+} were added to the incubation media. For measurement of the dienic conjugate contents mitochondrial lipids were extracted by hexane – isopropanol (1:1 v/v) mixture (9 ml per 1 ml of the sample) by shaking. After shaking 1 ml H_2O was added to the mixture to stratify hexane and isopropanol phases. Measurement of dienic conjugate contents was made in the hexane phase at 233 nm on spectrophotometer "SF-46" ("LOMO", USSR). The dienic conjugate contents in the sample were calculated according to 233 nm molar extinction coefficient to polyunsaturated fatty acids conjugated dienes $2{,}2 \times 10^5 \times M^{-1}\ sm^{-1}$[25].

Winter wheat shoots samples (3 g) were infiltrated by 1) Water, 2) Pyruvate (40 mM), 3) Linoleic acid (40 mM) and 4) CSP 310 (1 mg/mL) for 1 h and were packed in paper containers for stress treatment. Cold stress of winter wheat shoots was performed in thermostat at -4°C for 1 h. The control variant was not placed in stress conditions.

The oxygen uptake of winter wheat shoots was analyzed polarographically at 27°C using a platinum electrode of a closed type in a 1.4 mL volume cell. The reaction mixture contained 175 mM KCl and 25 mM Tris-HCl (pH 7.4).

All the experiments were made in six preparations. The data obtained were analyzed statistically, i.e. arithmetic means and standard errors were determined.

Abbreviations

BSA – bovine serum albumin, PUMP – plant uncoupling mitochondrial protein, AOX – alternative cyanide-resistant oxidase, CSP 310 – cold shock protein with molecular weight 310 kD.

Acknowledgements

The work has been performed, in part, with the support of the Russian Foundation of Basic Research (project 00-04-48093).

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