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REGULAR PAPER

Variety-specific response of wheat (*Triticum aestivum* L.) leaf mitochondria to drought stress

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Abstract The main objective of the present work was to examine leaf respiratory responses to dehydration and subsequent recovery in three varieties of winter wheat (*Triticum aestivum* L.) known to differ in their level of drought tolerance. Under dehydration, both total respiration and salicylhydroxamic acid (SHAM)-resistant cytochrome (Cyt) pathway respiration by leaf segments decreased significantly compared with well-watered plants. This decrease was more pronounced in the drought-sensitive Sadovo and Prelom genotypes. In contrast, the KCN-resistant SHAM-sensitive alternative (Alt) pathway became increasingly engaged, and accounted for about 80% of the total respiration. In the drought-tolerant Katya variety, increased contribution of the Alt pathway was accompanied by a slight decrease in Cyt pathway activity. Respiration of isolated leaf mitochondria also showed a variety-specific drought response. Mitochondria from drought-sensitive genotypes had low oxidative phosphorylation efficiency after dehydration and rewatering, whereas the drought-tolerant Katya mitochondria showed higher phosphorylation rates. Morphometric analysis of leaf ultrastructure revealed that mitochondria occupied approximately 7% of the cell area in control plants. Under dehydration, in the drought-sensitive varieties this area was reduced to about 2.0%, whereas in Katya it was around 6.0%. The results are discussed in terms of possible mechanisms underlying variety-specific mitochondrial responses to dehydration.

Keywords Drought stress · Leaf ultrastructure · Plant mitochondria · Wheat variety

Abbreviations

ADP/O	Ratio of the amount of phosphorylated ADP to oxygen consumed
AOX	Alternative oxidase
Alt pathway	Alternative pathway
Cyt pathway	Cytochrome pathway
PG	*n*-Propyl gallate
RC	Respiratory control
ROS	Reactive oxygen species
SHAM	Salicylhydroxamic acid

Introduction

Drought is one of the most significant abiotic stresses limiting plant production worldwide. Cultivating drought-tolerant crop varieties is a highly relevant strategy for achieving efficient and sustainable crop production systems (Erdei et al. 2002). Drought tolerance is controlled at different levels of organisation (Atkin and Macherel 2009; Shinozaki and Yamaguchi-Shinozaki 2007; Shinozaki et al. 1998). At a subcellular level, mitochondria participate in some metabolic processes involved in plant cell adaptation to dehydration (Pastore et al. 2007). Mitochondrial function seems to be a sensitive stress target, varying in relation to the substrate oxidised and the stress level (Flagella et al. 2006). The plant mitochondrial electron transport chain

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branches at the level of ubiquinone into a cytochrome (Cyt) pathway that generates the proton-motive force used for ATP synthesis, and an alternative (Alt), non-phosphorylating pathway that channels electrons directly to oxygen through Alt oxidase (AOX). Contribution of the Alt pathway to overall mitochondrial electron transport is affected by a number of factors, including plant specificities, developmental stage and environmental conditions (Hong et al. 2005; Maxwell et al. 1999; Noguchi et al. 2005; Noguchi and Terashima 2006). The limited number of reports dealing with mitochondrial respiration and electron partitioning under dehydration give inconclusive results. Using an oxygen-isotope-fractionation technique, Ribas-Carbo et al. (2005) showed that in soybean leaves under water stress, the Alt pathway is up-regulated, accompanied by a concomitant decrease in the activity of the Cyt pathway. Similarly, in wheat leaves, drought enhanced the rate of AOX-dependent oxygen uptake but the maximum capacity of the Cyt pathway was not affected (Bartoli et al. 2005). A study based on the titration of specific inhibitors of both respiratory pathways revealed a significant decrease in Cyt pathway activity under water stress without an effect on the Alt pathway (Gonzalez-Meler et al. 1997). Using the same approach, Zagdanska (1995) observed increased activity of the Cyt pathway in wheat.

Drought stress causes specific changes in plant cell ultrastructure, including plasmolysis, chromatin condensation, irregular shape of chloroplasts, a decreased amount or lack of starch grains, accumulation of plastoglobuli and swollen thylakoids (Munné-Bosch et al. 2001; Olmos et al. 2007). Zellnig et al. (2004) reported changes in mitochondrial morphology and decreased volume of drought-treated mitochondria in spinach leaves. In contrast, Berlin et al. (1982) observed an increase in the volume of mitochondria per palisade cell in drought-stressed leaves compared to nonstressed leaves of field-grown cotton (*Gossypium hirsutum* L.). This increase is a result of the larger size of individual mitochondria, as the mitochondrial number remains the same. It remains unclear whether variations in mitochondrial energy production dictate changes in mitochondrial configuration or vice versa.

The main objective of this work was to compare drought-induced structural and functional responses of leaf mitochondria from three wheat varieties with different tolerance to dehydration. Mitochondrial energetic and ultrastructural alterations were tested for their degree of reversibility. Our previous study on eight wheat varieties has shown variety-dependent changes in some biochemical and structural parameters under water stress (Simova-Stoilova et al. 2006). We selected three of these varieties—Sadovo, Katya and Prelom—for further investigation on the basis of their drought susceptibility. Despite the fact that, under field conditions, Katya is the most drought-tolerant wheat variety in Bulgaria (Kalapos et al. 1996), so far there are no studies pointing to any mechanisms involved in drought stress response and tolerance.

Materials and methods

Plant material and growth conditions

Three varieties of winter wheat (*Triticum aestivum* L.) differing in their field drought tolerance were selected for examination: Sadovo variety (the most widely distributed in Bulgaria, and considered less drought-tolerant and more susceptible to water stress), Katya (the most drought-tolerant Bulgarian variety; Kalapos et al. 1996) and Prelom (susceptible to water stress; Yordanov et al. 2001). Plants were grown in pots containing an enriched soil mixture (12 plants per pot) under a light intensity of 150 μmol m^{-2} s^{-1}, temperature between 25 and 21°C, and a 16-h photoperiod. Drought was induced on 8-day-old plants by withholding watering for 7 days, followed by a 3-day rehydration period. The degree of drought stress was determined as described in previous reports (Simova-Stoilova et al. 2006; Demirevska et al. 2008).

Leaf disk respiration measurements

Rates of oxygen uptake by leaf disks were measured polarographically with a Clark-type electrode (Hansatech, Norfolk, UK) at 25°C in 2.5 ml air-saturated solution containing 50 mM HEPES, 10 mM MES (pH 6.6) and 0.2 mM $CaCl_2$, according to the method of Noguchi and Terashima (1997). Leaf segments were sampled 6 h into the photoperiod. To prevent any increase in respiration due to slicing, the leaf disks were washed several times in the buffer solution before taking measurements. A piece of nylon netting was used to keep the segments above the stirrer bar. KCN (1 mM; from a 1 M stock in 20 mM HEPES) and 10 mM salicylhydroxamic acid [SHAM; 1 M stock in dimethyl sulfoxide (DMSO)] were used to assess the capacity of the Cyt and Alt pathways. As respiration of leaf segments was almost fully inhibited by a combination of these inhibitors, the exogenous application of inhibitors was taken to be effective. Recordings in the presence of inhibitors were performed after 30 min exposure to inhibitor treatment in a stirred oxygen electrode chamber. Incubation of leaf segments for 30 min in the absence of inhibitors resulted in only a slight (~6%) decrease in respiration rate. The respective solvent without the inhibitor was added to control samples. Total leaf respiration was measured without any inhibitor treatment. Oxygen uptake rates were calculated assuming that the concentration of oxygen in air-saturated buffer at 25°C is 240 μmol l^{-1}.

Isolation of mitochondria and respiratory measurements

Mitochondria were isolated by differential centrifugation according to Keech et al. (2005) with minor modifications. Briefly, leaves were ground in a medium consisting of 0.3 M sucrose, TES (pH 7.6), 2 mM EDTA, 10 mM KH_2PO_4, 25 mM TSPP, 1 mM glycine, 1% (w/v) PVP-40, 1% (w/v) essentially fat-free bovine serum albumin (BSA) and 50 mM Na ascorbate (added prior to grinding). The homogenate was filtered and centrifuged at 2,000 *g* for 5 min. The supernatant was additionally centrifuged at 15,000 *g* for 20 min. The pellet obtained was suspended in wash buffer (0.3 M sucrose, 10 mM TRIS pH 7.5, 10 mM KH_2PO_4 and 2 mM EDTA) and centrifuged at 15,000 *g* for 20 min. The resulting pellet containing the crude mitochondria was finally resuspended in a small volume of wash buffer. The quality of the isolated mitochondria was checked routinely by measuring cytochrome *c*-dependent oxygen uptake as previously described (Lee et al. 1994), and additionally examined by transmission electron microscopy.

Oxygen consumption was determined by incubating mitochondria in 1.2 ml reaction medium containing 0.3 M sucrose, 10 mM Tris (pH 7.5), 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1% (w/v) essentially fat-free BSA at 25°C. Respiratory control (RC) and the ratio of the amount of phosphorylated ADP to oxygen consumed (ADP/O) were determined according to Chance and Williams (1956) in the presence of malate (5 mM) plus glutamate (5 mM), succinate (10 mM), proline (10 mM) or NADH (1 mM), either with or without ADP (0.15 mM). The capacities of Cyt and Alt pathways were measured in the presence of 1 mM NADH plus 5 mM succinate as substrates. Cytochrome pathway respiration was measured according to Armstrong et al. (2006) with $CaCl_2$ (1.0 mM), pyruvate (5 mM), ADP (0.8 mM), 30 μM *n*-propyl gallate (PG) or 2 mM SHAM, and in the presence and absence of the uncoupling agent 2,4-dinitrophenol (15 μM). Alt pathway respiration was optimized by the addition of the Cyt pathway inhibitors antimycin (5 μM) or KCN (1 mM), and the AOX activators DTT (2 mM) and pyruvate (5 mM). All oxygen consumption rates are expressed as nmol min^{-1} O_2 mg^{-1} protein. Total soluble protein content was determined by the method of Bradford (1976) using BSA as a standard.

Transmission electron microscopy and morphometric analysis

Leaf sections or the pellet of isolated mitochondria were fixed with 2.5% glutaraldehyde in 30 mM PIPES-KOH buffer (pH 7.2) for 2 h, washed twice in 50 mM PIPES-KOH buffer (pH 6.8) for 30 min and postfixed in the same buffer with 1% OsO_4 for 2 h at 4°C. The samples were then washed several times, stained in 1% uranyl acetate (pH 5.5), dehydrated through an ethanol series (10–100%) followed by propylene oxide, then infiltrated in a mixture of Spurr's resin and propylene oxide (1/1, v/v), and finally embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with an transmission electron microscope (Jeol JEM 1010) operating at 100 kV. The morphometric analysis was performed using the software ImageJ (version 1.41o, National Institutes of Health, Bethesda, MA).

Statistical analyses

The results presented are the means with standard errors of 5–15 replicates from three independent experiments. Each replicate included four to five successive additions of ADP. One-way ANOVA followed by post hoc multiple comparison using Tukey's test (Sokal and Rohlf 1981) was applied to identify significant differences between the varieties.

Results

Several leaf respiratory characteristics were evaluated to compare responses to dehydration in three wheat genotypes: Sadovo, Katya and Prelom (Table 1). Drought level was monitored in terms of leaf water deficit, as described previously (Simova-Stoilova et al. 2006; Demirevska et al. 2008). In brief, cessation of watering for 7 days resulted in a 56–58% reduction in soil humidity. The leaf water deficit remained unchanged during the first 4 days, increased sharply afterwards, reaching 55–60% (severe water stress) on the 7th day of water deprivation. In the drought-sensitive wheat varieties, pronounced increases in proteolytic activity and membrane damage are found under such conditions (Simova-Stoilova et al. 2006).

The total rates of oxygen consumption by leaf segments from control plants did not vary significantly among the various genotypes (Table 1). Under dehydration in the drought-sensitive Sadovo and Prelom varieties consumption rates decreased on average 35%, and less than 20% in the drought-tolerant Katya, compared to well-watered control plants. The capacity of the Alt pathway was assessed by measuring KCN-resistant and SHAM-sensitive oxygen uptake by leaf segments (Lambers et al. 1983). In preliminary experiments, the mean respiration rates of leaf disks at different SHAM concentrations (1–25 mM) were plotted against those obtained in the presence of both SHAM and KCN. This plot showed that 10 mM SHAM and 1 mM KCN were the minimum concentrations able to elicit a maximum effect. Concentrations of SHAM higher

Table 1 Respiratory characteristics of leaf segments from three wheat varieties grown under normal watering, drought, and after recovery

Variety	Total respiration (V_t) (nmol O_2 g^{-1} dry mass s^{-1})	SHAM-resistant respiration (V_{cyt}) (nmol O_2 g^{-1} dry mass s^{-1})	KCN-resistant SHAM-sensitive respiration (V_{alt}) (nmol O_2 g^{-1} dry mass s^{-1})	Residual respiration (V_{res}) (nmol O_2 g^{-1} dry mass s^{-1})
Sadovo				
Control	23.8 a	16.0 a	9.4 a	3.2 a
Drought	15.5 b	2.6 b	12.9 b	1.9 a
Recovery	18.9 b	10.6 c	11.3 b	2.3 a
Katya				
Control	21.9 a	15.3 a	9.1 a	2.5 a
Drought	18.0 b	9.0 c	13.6 b	1.9 a
Recovery	23.7 a	12.6 c	14.3 b	3.2 a
Prelom				
Control	20.9 a	15.2 a	7.4 a	2.6 a
Drought	14.1 b	2.7 b	11.8 b	1.7 a
Recovery	17.7 b	8.9 c	11.9 b	2.3 a

Respiration was measured in the absence of inhibitors or in the presence of 1 mM KCN, 10 mM salicylhydroxamic acid (SHAM), or both KCN plus SHAM. Residual respiration was determined in the presence of inhibitors of both pathways. Different letters in the same column indicate significant difference by multiple comparison Tukey's test ($P < 0.05$)

than 20 mM caused inhibition of Cyt pathway activity as well (data not shown).

In control plants, the Cyt pathway was responsible for approximately 70% of the total respiration in all varieties studied (Table 1). Under dehydration, in the drought-susceptible genotypes the Cyt pathway displayed a trend toward being very sensitive to water cessation, and its contribution to total respiration was less than 20%. On the other hand, the Alt pathway became increasingly engaged and accounted for about 80% of total respiration. In leaf segments from the drought-tolerant Katya variety, Cyt pathway activity was also decreased slightly but it still accounted for about 50% of total respiration. Residual respiration in the presence of both respiratory inhibitors was drought- and variety-insensitive (Table 1).

Drought effects on wheat leaves were further investigated by comparing respiratory characteristics of isolated leaf mitochondria (Table 2). The procedure by Keech et al. (2005) for extraction of a highly functional crude mitochondrial fraction allowed us to obtain intact leaf mitochondria with excellent respiratory coupling persisting for at least several hours after isolation. Examination of mitochondrial outer membrane integrity showed intactness of 95–100% ($n = 6$) for control and recovered plants, and $93 \pm 3\%$ ($n = 5$) for drought-stressed plants. Addition of 0.15 mM ADP to the electrode reaction chamber greatly increased mitochondrial oxygen uptake (state 3), which was also indicative of the good functional state of the isolated organelles (Table 2). When the added ADP was fully phosphorylated, respiration rates decreased (state 4). The high structural integrity and lack of substantial contamination with other organelles were additionally verified by transmission electron microscopy (Fig. 1).

As shown in Table 2, state 3 and 4 respiration rates (expressed on a protein basis) of mitochondria isolated from control leaves were not significantly different among the varieties. Mitochondria achieved adequate respiratory coupling with all substrates tested, and the RC ratio was in the range 1.6–3.2. The ADP/O ratio, considered as an index of phosphorylation efficiency, reached maximum values in the presence of malate-glutamate and varied between 2.4 and 2.7. Oxygen consumption at normal watering with succinate plus NADH as substrates was considerably increased (Table 3) as compared to the rates with single substrates (Table 2), but lower than the sum of individual respiration rates. Estimation of the electron flux through both respiratory pathways on a mitochondrial protein basis showed approximately twice higher capacity of the energy-producing Cyt pathway in control mitochondria (Table 3). The ADP/O ratio ranged between 1.6 and 1.8, indicating that the cooperative oxidation of two substrates was coupled with two proton-extrusion sites.

Under water stress conditions, mitochondria from all varieties presented with a higher rate of state 3 respiration (Table 2) than controls. However, in Sadovo and Prelom varieties, state 4 respiratory activity was increased nearly twofold, which was expressed in reduced RC and ADP/O ratios. The only exception was proline-supported oxygen consumption, where state 3 respiratory rates were greatly decreased and the RC and ADP/O ratios were close to being unmeasurable. Drought treatment increased capacity of the Alt pathway, changing its contribution from 30–40%

Table 2 Respiratory characteristics of mitochondria isolated from leaves of different wheat varieties grown under normal watering (C), drought (D), and after recovery (R) in the presence of 5 mM malate + 5 mM glutamate, 10 mM succinate, 10 mM proline, and 1 mM NADH. Substrate oxidation rates are expressed as nmol O_2 min^{-1} mg^{-1} protein

Substrates	Sadovo			Katya			Prelom		
	C	D	R	C	D	R	C	D	R
Malate + glutamate									
State 3	77.4 a	95.4 b	84.5 ab	84.2 ab	79.4 a	77.3 a	82.1 ab	97.1 b	79.6 a
State 4	26.7 a	63.6 b	44.5 c	33.7 a	34.5 a	30.9 a	25.7 a	53.9 bc	39.9 c
RC	2.9 a	1.5 b	1.9 b	2.5 a	2.3 ab	2.5 a	3.2 a	1.8 b	2.0 b
ADP/O	2.6 a	1.1 b	1.5 b	2.4 a	2.0 a	2.1 a	2.7 a	1.5 b	1.6 b
Succinate									
State 3	71.1 a	83.9 b	73.7 a	72.9 a	64.1 a	69.8 a	69.3 a	74.8 a	70.2 a
State 4	37.4 a	69.8 b	49.8 a	42.9 a	45.8 a	43.6 a	43.4 a	68.1 b	54.0 ab
RC	1.9 a	1.2 b	1.5 ab	1.7 a	1.4 b	1.6 ab	1.6 ab	1.1 b	1.3 b
ADP/O	1.5 a	1.1 b	1.3 ab	1.6 a	1.2 ab	1.4 a	1.5 a	1.0 b	1.1 b
Proline									
State 3	65.7 a	48.2 b	70.2 a	79.6 a	68.2 a	87.6 a	67.1 a	43.7 b	66.5 a
State 4	27.4 a	39.4 b	35.0 ab	31.1 a	29.7 a	35.3 ab	33.7 ab	39.7 b	41.6 b
RC	2.4 a	1.2 b	2.0 a	2.6 a	2.3 a	2.5 a	2.0 a	1.1 b	1.6 ab
ADP/O	2.0 a	1.0 b	1.7 a	2.1 a	1.9 a	2.1 a	1.7 a	1.0 b	1.2 b
NADH									
State 3	56.8 a	66.7 b	61.3 ab	69.7 b	71.2 b	70.2 b	58.6 a	72.9 b	63.4 ab
State 4	28.3 a	55.6 b	40.2 ab	36.6 a	47.5 b	41.3 ab	27.9 a	52.1 b	40.2 ab
RC	2.0 a	1.2 b	1.5 ab	1.9 a	1.5 ab	1.7 a	2.1 a	1.4 ab	1.6 ab
ADP/O	1.7 a	1.0 b	1.1 b	1.6 a	1.4 a	1.4 a	1.6 a	1.1 b	1.2 b

Different letters in the same row indicate significant difference by multiple comparison Tukey's test ($P < 0.05$)

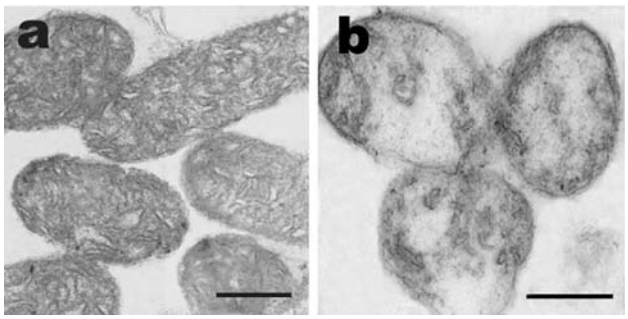

Fig. 1 Transmission electron micrograph of mitochondria isolated from leaves of winter wheat (*Triticum aestivum* L.), variety Katya, grown under normal watering (**a**) and drought (**b**). *Bar* 0.5 μm

in controls to 60–65% in drought-exposed plants (Table 3). In leaves of drought-sensitive varieties, this increase was accompanied by a substantial decrease in Cyt pathway capacity. In this way, the capacity of the Alt pathway exceeded approximately twice the Cyt pathway capacity, which resulted in a very low ADP/O ratio. Measurement of the ADP/O ratio in the presence of SHAM and PG during the oxidation of succinate plus NADH additionally confirmed the reduction in the efficiency with which mitochondria synthesise ATP. As with the leaf segment samples, mitochondria isolated from Katya variety showed an adequate RC and phosphorylation efficiency.

To define the extent of mitochondrial damage and the ability to recover, drought treatment was followed by a 3-day period of rehydration. The oxidative activity of Sadovo and Prelom mitochondria was partly recovered, resulting in higher RC and ADP/O ratios (Table 2), as compared to drought-exposed mitochondria. Cyt pathway capacity showed modest recovery and did not reach control values (Table 3). Complete reversibility of Cyt pathway capacity was noted only for the drought-tolerant Katya variety. On the other hand, Alt pathway capacity stayed higher than the control rates for all varieties.

Transmission electron microscopy observations showed quite variable morphology and distribution of leaf mitochondria in control, drought-treated and recovered plants (Fig. 2a–i). Morphometric analysis revealed that, in control leaves, the mean size of these organelles was about 0.38 μm^2 (Fig. 3a) and they occupied approximately 7% of the total cell area (Fig. 3b) as about half of them had an elongated shape (Fig. 3c). The electron-dense matrix and clearly visible numerous cristae were also typical features of the control mitochondria (Fig. 2a–c). Drought treatment caused loss of internal mitochondrial structure for all three

Table 3 Oxygen uptake rates and respiratory capacity of the cytochrome (Cyt) and alternative (Alt) pathway in mitochondria isolated from leaves of three wheat varieties grown under normal watering, drought, and after recovery

Variety	State 3 (NADH + suc)	RC ratio	ADP/O	Cyt pathway capacity	Alt pathway capacity	ADP/O (NADH + suc + SHAM + PG)
Sadovo						
Control	124 ± 7 a	2.0 a	1.8 a	94 ± 12 a	47 ± 4 a	1.7 a
Drought	148 ± 8 b	1.4 b	1.2 b	52 ± 9 b	96 ± 10 b	1.1 b
Recovery	145 ± 11 b	1.5 b	1.4 b	68 ± 8 b	85 ± 11 b	1.4 b
Katya						
Control	135 ± 9 a	2.0 a	1.8 a	105 ± 11 a	42 ± 6 a	1.7 a
Drought	154 ± 8 b	1.6 ab	1.5 ab	86 ± 9 a	92 ± 9 b	1.5 ab
Recovery	158 ± 9 b	1.8 ab	1.6 a	99 ± 8 a	86 ± 7 b	1.6 a
Prelom						
Control	120 ± 7 a	1.9 a	1.6 a	85 ± 9 a	38 ± 8 a	1.6 a
Drought	139 ± 9 b	1.3 b	1.1 b	51 ± 2 b	88 ± 6 b	ND
Recovery	128 ± 11 ab	1.4 b	1.3 b	58 ± 8 b	75 ± 12 b	1.3 b

Data are expressed in nmol O_2 mg protein^{-1} min^{-1}. Different letters in the same column indicate significant difference by multiple comparison Tukey's test ($P < 0.05$)

ND Not determined

varieties (Fig. 2d–f). In Sadovo and Prelom the relative area of the cells occupied by mitochondria was reduced to about 2.0%, whereas in Katya this area was still maintained at 6.0% (Fig. 3b). In drought-treated leaves of all varieties, the proportion of spherial and oval-shaped mitochondria was greatly increased (Fig. 3c). These organelles exhibited a swollen morphology (Fig. 2d–f) and significantly larger average size (about 0.57 μm^2) than in the controls (Fig. 3a). It should also be noted that, after the rehydration period, the ultrastructure of the Katya mitochondria recovered completely, including the predominant elongated shape (Fig. 3c), whereas in the drought-susceptible genotypes, some typical damage symptoms could still be seen (Fig. 2g–i).

Discussion

This work provides information on the effect of dehydration on leaf mitochondrial respiration, combining studies at tissue and organelle levels. The use of three wheat genotypes with different drought tolerance allowed clear evidence for a variety-specific mitochondrial reaction to water cessation and recovery to be obtained. In general, in fully expanded mature leaves, the respiratory response to dehydration is quite variable, ranging from a decrease or no effect, to stimulation (reviewed in Atkin and Macherel 2009). In our study, respiration rates (measured as oxygen consumption by leaf segments and expressed on a dry mass basis) decreased slightly but significantly in all three varieties studied. This reduction was more pronounced in the drought-sensitive varieties Sadovo and Prelom. The activity of the Cyt pathway showed a similar tendency, as its contribution to total respiration decreased from approximately 70% in control leaves to 20% in dehydrated leaves of drought-sensitive varieties. In contrast, the proportion of respiration through the nonphosphorylating Alt pathway increased to represent more than 80% of total respiration. In the drought-tolerant Katya variety, however, the contribution of the Cyt pathway did not decrease dramatically, and under dehydration equalled that of the Alt pathway. It could be concluded that the observed decrease in total respiration under dehydration was attributable mostly to the reduced activity of the Cyt pathway, which could not be compensated for by the increased use of Alt pathway respiration. Generally, any decrease in the contribution of the Cyt pathway implies a lower ATP yield per oxygen consumed (Gonzalez-Meler et al. 2001). This suggests that the dehydration-induced reduction in respiration may impair ATP-requiring processes such as sucrose synthesis, phloem loading and tissue maintenance (Atkin and Macherel 2009).

Comparison of the respiratory characteristics of isolated leaf mitochondria also provided information on the variety-specific dehydration response of these organelles. Mitochondria from the drought-sensitive Sadovo and Prelom varieties were negatively affected, and this effect clearly persisted after their isolation from drought-treated leaves. Despite the higher state 3 oxidation rates, the degree of mitochondrial respiratory coupling in drought-sensitive varieties was considerably decreased as compared to the well-watered control and drought-tolerant Katya. The greater substrate-oxidizing ability of drought-treated mitochondria remained, in contrast to the decreased

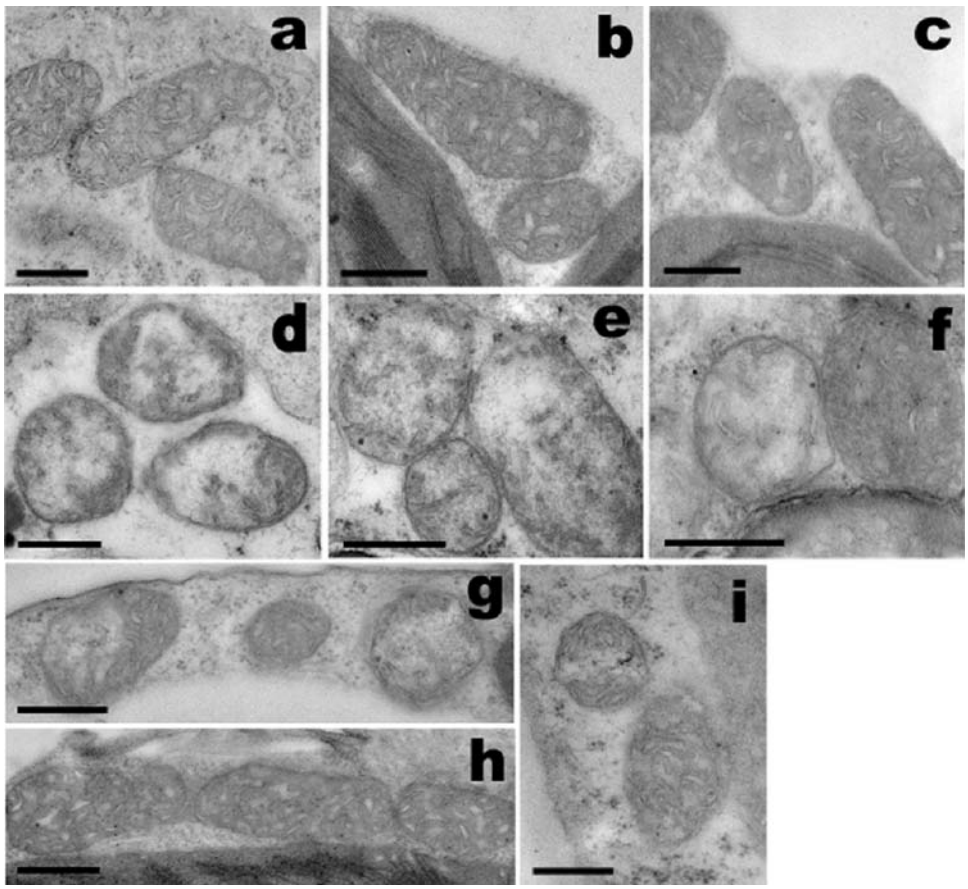

Fig. 2 Transmission electron micrographs of leaf mitochondria from wheat plants, varieties Sadovo (**a**, **d**, **g**), Katya (**b**, **e**, **h**) and Prelom (**c**, **f**, **i**), grown under normal watering (**a**–**c**), drought (**d**–**f**) and after recovery (**g**–**i**). *Bar* 0.5 μm

mitochondrial respiration exhibited by dehydrated leaf segments. One possible explanation for this disparity is that the respiratory measurements on isolated mitochondria were performed with saturating ADP and substrates, whereas in intact leaf segments, respiration could be ADP- and substrate-limited. Limitations in ADP supply in intact tissues generally have a greater detrimental effect on Cyt pathway activity, due to its coupling to proton translocation and ATP synthesis. Additional evidence for ADP and substrate limitation in drought-sensitive varieties comes from the lower reduction in the contribution of Cyt pathway activity in isolated mitochondria than in intact tissue. SHAM-resistant respiration of isolated organelles represented more than 40% of total respiration, whereas in leaf segments it declined to less than 20%. It is thought that when Cyt pathway activity is restricted or saturated, the Alt pathway becomes more active. Under severe water stress, soybean leaves show a decrease in the activity of the Cyt pathway concomitant with a very significant increase in use of the Alt pathway (Ribas-Carbo et al. 2005). However, our results showed that the response of the Cyt pathway to drought did not necessarily correlate with the response of the Alt pathway. In drought-sensitive varieties, the activity of the Cyt pathway was inhibited strongly by dehydration, and the activity of the Alt pathway increased concomitantly. However, in the drought-resistant Katya genotype, the sharp increase in Alt pathway activity did not correlate with a proportional decrease in Cyt pathway respiration.

It should also be noted that, in some drought-tolerant species, besides active AOX, mitochondria possess two additional energy-dissipating systems: an ATP-sensitive potassium channel ($PmitoK_{ATP}$) and a highly active uncoupling protein (UCP) (Pastore et al. 2007). Both of these play a key role as defence systems under environmental stress, as they are able to dampen mitochondrial reactive oxygen species (ROS) production (Fratianni et al. 2001; Trono et al. 2004). Pastore et al. (2007) found that moderate osmotic or salt stress applied to seedlings resulted in the activation of $PmitoK_{ATP}$ and UCP, suggesting mitochondrial acclimatisation to drought stress. It is tempting to speculate that, under our experimental conditions, these two dissipating systems could be also involved in the dehydration-induced mitochondrial response. Mitochondria from the drought-sensitive Sadovo and Prelom varieties displayed a very low ADP/O ratio even when the AOX pathway was inhibited by SHAM and/or PG. This implies operation of other uncoupling systems.

The observed inhibition of proline-supported mitochondrial oxidation under drought treatment is consistent

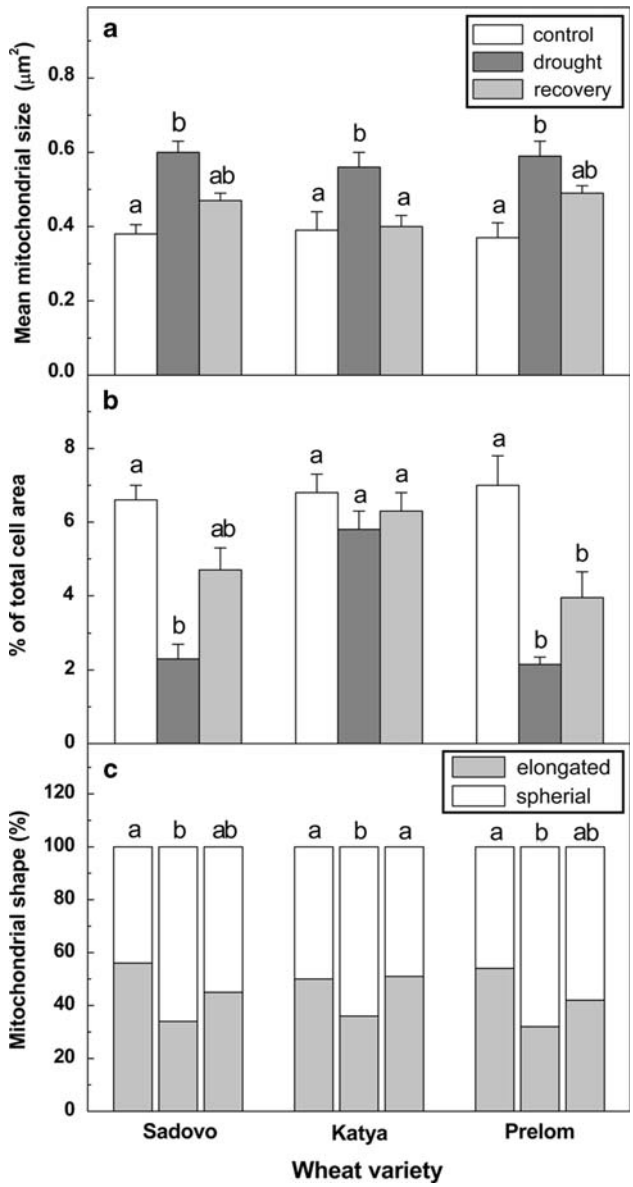

Fig. 3 Quantitative morphometric analysis of ultrathin sections of mesophyll cells in winter wheat leaves. **a** Mean mitochondrial size, **b** cell area occupied by mitochondria, and **c** mitochondrial shape under normal watering, drought and after recovery are calculated. In **b**, the area measurements are expressed as a percentage of the total cell volume. The means denoted by the different *lower case letters* indicate significant difference by multiple comparison Tukey's test ($P < 0.05$). In **c**, different *lower case letters* mean significant difference in both shape populations

with the important role of proline as a compatible osmolyte (reviewed in Atkin and Macherel 2009). Generally, proline accumulates during water stress in response to drought and rapidly disappears upon recovery. Flagella et al. (2006) observed inhibition of proline-dependent oxygen uptake and phosphorylation efficiency in durum wheat seedlings even under moderate stress conditions. The inhibition of proline-dependent ATP synthesis may be an adaptation mechanism leading to proline accumulation. Thus, mitochondria may not only be a target of stress, but may also play a key role in counteracting environmental stresses and determining stress resistance by modulating cell redox homeostasis.

The effect of dehydration on mitochondria from drought-sensitive varieties was partly reversible. Despite the fact that, even after the 3-day recovery period, they could not reach the level of oxidative activity of the controls, respiratory ATP production was still partly restored. This is in agreement with the findings of Taylor et al. (2005), who showed that drought induces mostly defence reactions and causes less protein damage than other kinds of stress, such as cold or herbicide treatments.

Ultrastructural examination by transmission electron microscopy of leaf samples from all three varieties displayed similar visible symptoms of senescence upon exposure to drought. The relatively stable respiration and phosphorylation efficiency of Katya, however, showed that, despite visible damage, its mitochondria were still able to maintain reasonable function. Quantitative morphometric analysis provides a useful tool for converting the visual structural alterations into numerical data (Zellnig et al. 2004). An interesting result was that, upon drought treatment, the relative area of leaf cells occupied by mitochondria was significantly reduced only in the drought-sensitive genotypes, whereas in the drought-tolerant variety it was close to the controls. The relative structural stability of the mitochondria from Katya could be considered an additional advantage, contributing to its drought tolerance. In addition, dehydration affected the morphology of mitochondria, changing their shape from predominantly elongated to spherical and oval. After the 3-day recovery period, only the organelles of the drought-tolerant genotype were fully restored and resembled the control ultrastructure. The occurrence of differently formed mitochondria could be a result of their different energy status. Skubatz and Kunkel (2000) reported that, in *Sauromatum guttatum*, mitochondrial morphology changes during periods of different respiratory activity. Other authors connect the altered external morphology of mitochondria to the rearrangement of cristae caused by changes in their conformational state (Tyler 1992; Logan and Leaver 2000). Our data showed a good correlation between the dehydration-induced respiratory changes and the variety-specific alterations in the cell area occupied by mitochondria. However, the observed modulation in mitochondrial shape did not correspond to the altered respiratory characteristics, as varieties with different drought sensitivity displayed similar alterations. On the other hand, Yoshinaga et al. (2005) reported that morphological changes in mitochondria are one of the early indicators of whether cells are affected by ROS stress.

Using various ROS-inducing chemicals, the same authors demonstrated dramatic morphological changes in mitochondria and cessation of cytoplasmic streaming, which might be a common feature of cells under different stress conditions. In addition, the observed mitochondrial swelling upon dehydration again raises the possibility that other energy-dissipating systems activated by ROS, such as $PmitoK_{ATP}$, are engaged upon drought stress. In view of the fact that $PmitoK_{ATP}$ is a major regulator of mitochondrial volume (Garlid and Paucek 2003), its participation in the adjustment of mitochondrial matrix volume to fluctuating plant water status seems a reasonable hypothesis. The mechanisms that regulate mitochondrial morphology are incompletely understood, but studies to date suggest that any changes in mitochondrial shape can have a profound impact on the functional output of these organelles and vice versa (reviewed in Soubannier and McBride 2009).

In conclusion, using three wheat genotypes, we have revealed distinct variety-specific respiratory responses to severe dehydration and subsequent recovery. Phosphorylation efficiency of the mitochondria in the drought-sensitive Sadovo and Prelom genotypes was significantly reduced under dehydration due to decreased Cyt pathway activity. These organelles also exhibited a reduced presence in leaf tissue under drought conditions. On the contrary, mitochondria from the drought-tolerant Katya variety showed higher phosphorylation rates and better structural performance. Whether the variety-specific responses are a consequence or a cause of plant drought tolerance remains a moot point. It could be suggested that sustained mitochondrial energy production in the Katya variety could underpin its higher drought tolerance capability, providing a solid base for overcoming the stress periods. Thus, leaf mitochondria represent an important cellular components that could confer a great advantage to plants within the same species in sustaining and overcoming drought periods. The recognised variety-specific peculiarities of mitochondrial functioning under drought could provide important clues to the successful design of transgenic plants with improved performance under dehydration.

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