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Monitoring the *in vivo* redox state of plant mitochondria: Effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge

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ABSTRACT

In animals, the impact of ROS production by mitochondria on cell physiology, death, disease and ageing is well recognised. In photosynthetic organisms such as higher plants, however, the chloroplast and peroxisomes are the major sources of ROS during normal metabolism and the importance of mitochondria in oxidative stress and redox signalling is less well established. To address this, the *in vivo* oxidation state of a mitochondrially-targeted redox-sensitive GFP (mt-roGFP2) was investigated in *Arabidopsis* leaves. Classical ROS-generating inhibitors of mitochondrial electron transport (rotenone, antimycin A and SHAM) had no effect on mt-roGFP oxidation when used singly, but combined inhibition of complex III and alternative oxidase by antimycin A and SHAM did cause significant oxidation. Inhibitors of complex IV and aconitase also caused oxidation of mt-roGFP2. This oxidation was not apparent in the cytosol whereas antimycin A + SHAM also caused oxidation of cytosolic roGFP2. Menadione had a much greater effect than the inhibitors, causing nearly complete oxidation of roGFP2 in both mitochondria and cytosol. A range of severe abiotic stress treatments (heat, salt, and heavy metal stress) led to oxidation of mt-roGFP2 while hyperosmotic stress had no effect and low temperature caused a slight but significant decrease in oxidation. Similar changes were observed for cytosolic roGFP2. Finally, the recovery of oxidation state of roGFP in mitochondria after oxidation by H_2O_2 treatment was dramatically slower than that of either the cytosol or chloroplast. Together, the results highlight the sensitivity of the mitochondrion to redox perturbation and suggest a potential role in sensing and signalling cellular redox challenge.

1. Introduction

Maintenance of a reduced intracellular environment is essential for the functioning of most biological processes and tight homeostasis of intracellular redox state is therefore critical. In aerobic organisms, the generation of reactive oxygen species (ROS) has the potential to perturb this homeostasis leading to oxidative stress and ultimately cell death [1]. Thus, aerobic organisms have evolved an extensive network of antioxidant enzymes and low-molecular weight antioxidants to buffer this oxidation [2,3]. The most important non-protein thiol-based antioxidant is the tripeptide glutathione [3,4]. Under non-stress conditions the glutathione pool is almost completely reduced, but detoxification of ROS by the ascorbate-glutathione cycle leads to transient glutathione oxidation. It has been proposed that this glutathione oxidation can be exploited by the cell as a signal of redox state [5–7].

In plant cells ROS are produced in multiple locations, including the mitochondrion, chloroplast, peroxisome and apoplast [8]. Membrane permeable and relatively long-lived ROS, such as hydrogen peroxide, may diffuse from these sites into the cytosol and be detoxified there [9]. However, mitochondria and chloroplasts have endogenous ascorbate and glutathione-based antioxidant systems and detoxification within organelles can also occur [10]. To date, the chloroplast has been the focus of most research, mainly because of the potential for this organelle to generate ROS at very high rates during photosynthesis and photoinhibition. Recently, though, it has become apparent that alterations in mitochondrial ROS production or electron transport can have far reaching consequences for the cell [11]. For example, mutation of complex I of the respiratory chain is accompanied by a general adjustment of cellular antioxidant systems and also impacts on cell death pathways [12,13]. In addition, mutation or knockdown of mitochondrial respiratory chain bypasses or antioxidant enzymes leads to growth and stress phenotypes [14–16]. In these examples it is not clear whether the observed changes are a result of oxidative damage or specific signals resulting from a perturbation of redox state. Discriminating between these two possibilities remains a major challenge in plant redox biology.

Given the increasing awareness of the importance of mitochondrial redox state for plant cells and the dual role of glutathione as a redox buffer and as a signal molecule, it would be helpful to have a better characterisation of the relationship between mitochondrial bioenergetic activity and the oxidation state of the glutathione pool. We have therefore used a redox-sensitive GFP (mt-roGFP2) targeted to *Arabidopsis* mitochondria [17,18] to make *in vivo* measurements of mitochondrial redox state. It has been previously shown that this probe responds rapidly to alterations in intracellular redox state of plant cells [18–20] and via its specific interaction with glutaredoxin,

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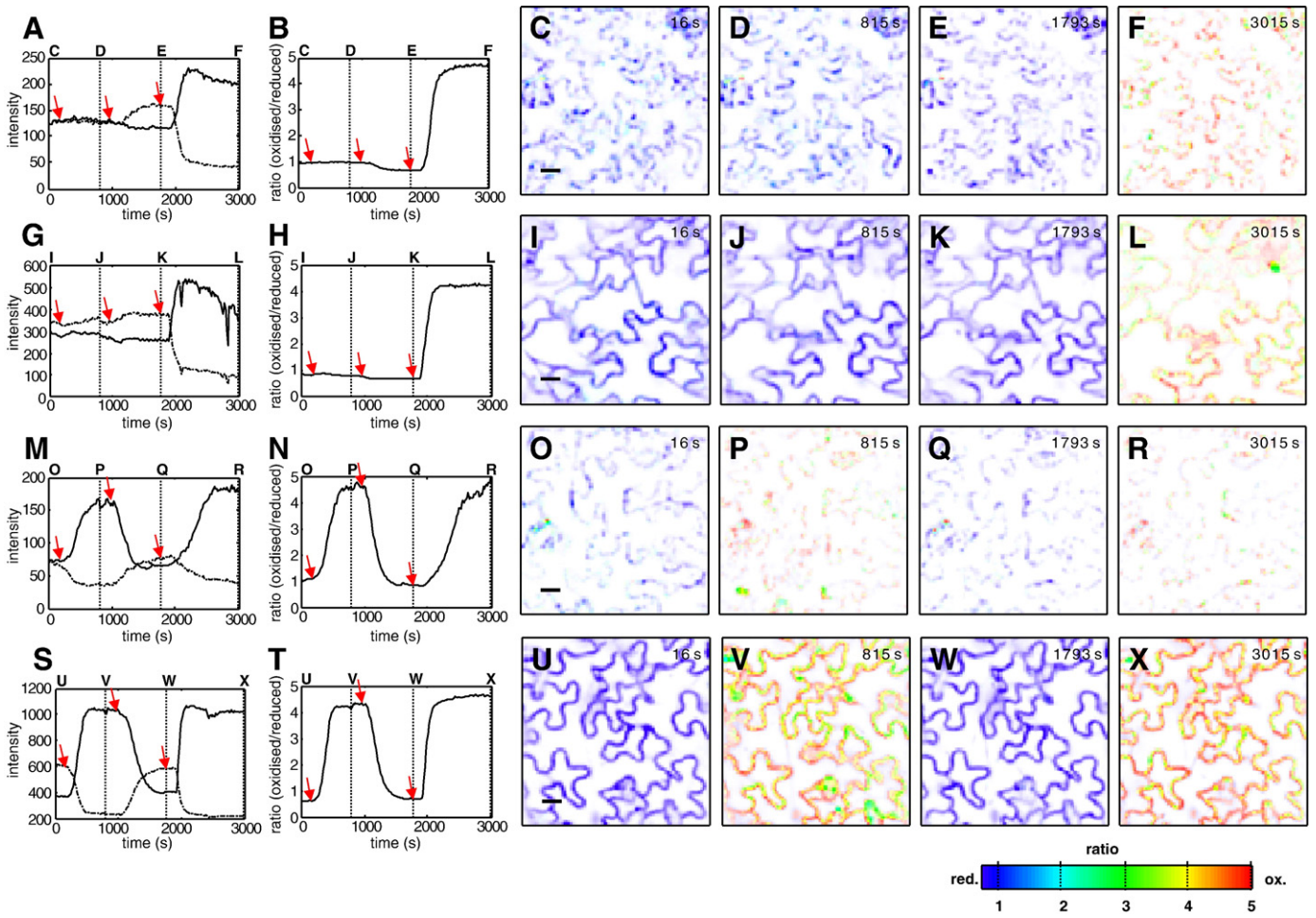

Fig. 1. roGFP2 expressed in mitochondria (mt-roGFP2) and cytosol (c-roGFP2) of *Arabidopsis* leaf epidermis cells shows rapid response to changes in redox state. *In-vivo* timecourse of mt-roGFP2 (A–F) and c-roGFP (G–L) behaviour followed by calibration using 10 mM DTT and 100 mM H_2O_2. Real time effect of 100 μM menadione on mt-roGFP2 (M–R) and c-roGFP2 (S–X) followed by calibration using 10 mM DTT and 100 mM H_2O_2. Red arrows indicate the changes of perfusion solution in the following sequence: treatment (menadione or control), DTT, H_2O_2. Panels in the first column (A, G, M, S) show the fluorescence intensities for both excitation wavelengths (405 nm —; 488 nm ---). Panels in the second column (B, H, N, T) show the corresponding ratio values. The time points of the corresponding images are marked by vertical dotted lines. Ratiometric images show resting state in column 3 (C, I, O, U), state after treatment in column 4 (D, J, P, V), state after reduction with DTT in column 5 (E, K, Q, W) and state after oxidation with H_2O_2 in column 6 (F, L, R, X). Scale bars = 10 μm. Timecourse perfusion experiments were replicated 3 times with similar results and a typical timecourse is shown.

reports on the oxidation of the glutathione pool [19]. Here we analyse the effects of application of a range of classical mitochondrial respiratory inhibitors and other chemicals on mt-roGFP2 oxidation. In addition, the effect of different abiotic stress treatments is assessed and the recovery of mitochondrial redox state after oxidative insult is compared to other subcellular compartments.

2. Materials and methods

2.1. Plant materials

RoGFP-expressing *Arabidopsis* lines employed in this study were in Columbia background and have been described previously (Schwarzländer et al. [18]). For all experiments surface sterilized seeds were plated on 0.7% agar plates supplemented with 1/2-strength Murashige and Skoog (MS) medium. Seedlings were grown in a controlled environment chamber with a 14/10 h day/night cycle (21/19 °C) at a light intensity of 50 μmol photons m^{-2} s^{-1}. Rosette leaves were used for the experiments from plants at an age of 20–25 days.

2.2. Inhibitor treatments and abiotic stress conditions

Chemicals were purchased from Sigma-Aldrich. Stock solutions of inhibitors (at least 500×) were prepared in water or ethanol. For treatments, stocks were diluted in 0.01% TWEEN or 1/2-strength MS medium (for perfusion experiments). Control solutions were supplemented with the appropriate amount of solvent. Seedlings were kept in darkness for 2 h prior to treatments and were submerged in, or excised leaves perfused with, inhibitor solution in the dark for the duration of the treatment.

For abiotic stress treatments, seedlings were submerged in 0.01% TWEEN containing the appropriate chemical in the light. Temperature treatments were applied to seedlings on agar plates in the light.

The following concentrations of inhibitors were used:

Inhibitor	Mitochondrial target	Stock solvent	Concentration in stock-solution	Final concentration
Antimycin A	Complex III	EtOH	50 mM	20 μM
Menadione	Redox active quinone	EtOH	100 mM	100 μM
Monofluoracetate (MFA)	Aconitase	–	–	10 mM
Rotenone	Complex I	EtOH	~25 mM	50 μM
Methyl viologen	Redox active quinone	Water	100 mM	100 μM
Potassium cyanide	Complex IV	Water	500 μM	500 μM
Salicylhydroxamic acid (SHAM)	Alternative oxidase	EtOH	1 M	2 mM
Sodium azide	Complex IV	Water	500 μM	500 μM

2.3. Respiratory measurements

Oxygen consumption by whole rosette leaves was measured in a Clark-type oxygen electrode. Assays were performed in the dark in 2 ml 0.01% TWEEN and inhibitors were applied as described in Section 2.2.

2.4. Confocal laser scanning microscopy imaging and image analysis

Confocal microscopy was carried on the abaxial side of rosette leaves using a Zeiss confocal microscope LSM510 META equipped with lasers for 405 nm and 488 nm excitation. Settings for imaging roGFP were chosen as described before (Schwarzlander et al. [18]). All imaging parameters were kept constant throughout all measurements. To collect single images, leaves were excised from seedlings after treatment and mounted abaxial side uppermost on a glass slide. For time-lapse imaging, leaf-samples were placed abaxial side uppermost in a perfusion chamber (RC-21BR, Warner Instruments LLC, Hamden, CT, USA), and immobilised with glass wool. Leaf pieces were perfused with treatment solutions and then calibrated using 10 mM DTT and 100 mM H_2O_2 as described in Schwarzlander et al. [18]. Ratiometric analysis of roGFP images and time series was carried out using a custom MatLab (The MathWorks, Nantick, MA) analysis suite as described previously (Schwarzlander et al. [18]).

2.5. Statistical analysis

Pairwise treatments were compared using the Students *t*-test algorithm in Microsoft Excel (Microsoft, USA). Significant differences ($p<0.05$) are labelled with asterisks. Errors are given as standard error of the mean.

3. Results

3.1. Effect of a range of inhibitors of mitochondrial respiration on roGFP oxidation state

We have previously established transgenic *Arabidopsis* lines with roGFP2 targeted to mitochondria or cytosol and demonstrated that the probe responds rapidly to redox changes in these locations [18]. To establish the baseline response of the probe in both compartments, excised leaves were perfused sequentially with 10 mM dithiothreitol and 100 mM H_2O_2 to generate fully reduced and oxidized forms, respectively. The probe localised in either mitochondria (Fig. 1A–F) or cytosol (Fig. 1G–L) responded rapidly, reaching stable reduced and oxidized states within 10 min of the solution change. To test the relationship between endogenous ROS and roGFP redox state, leaves were perfused with menadione as a positive control. Menadione is a redox-active quinone known to generate ROS in mitochondria [21]. Menadione caused rapid and almost complete oxidation of both mt-roGFP2 and c-roGFP2 (Fig. 1M–X).

Blockages of mitochondrial electron transport can increase the production of ROS. First we measured oxygen consumption to assess the impact of a number of classical mitochondrial inhibitors on respiration in *Arabidopsis* leaves (Fig. 2). Inhibitors were added in concentrations which have been reported to cause inhibition when applied exogenously [22–26]. In the control, the rate of oxygen consumption did not change (Fig. 2A). Antimycin A (inhibits complex III) did not cause any rate change within the first 20 min after application but the rate was reduced to less than a quarter after 2 h (Fig. 2B). Rotenone (inhibits complex I) did not result in any change of oxygen consumption rate within 2 h (the apparent slight rate increase in Fig. 2C was not significant), nor did SHAM (inhibitor of the alternative oxidase) within 20 min (Fig. 2D). A combination of antimycin A and SHAM decreased the rate steadily over time resulting in a practically complete blockage of oxygen uptake after 2 h (less than 10% of start rate, Fig. 2E). Treatments with KCN and azide (both inhibit complex IV, Fig. 2F, G) caused in an immediate reduction of oxygen consumption ($44\pm15\%$ and $47\pm4\%$ after 10 min respectively). No significant changes could be measured for monofluoracetate (inhibits aconitase, Fig. 2H) and methyl viologen (inhibits complex I and chloroplastic electron transport, Fig. 2I). Interestingly, menadione

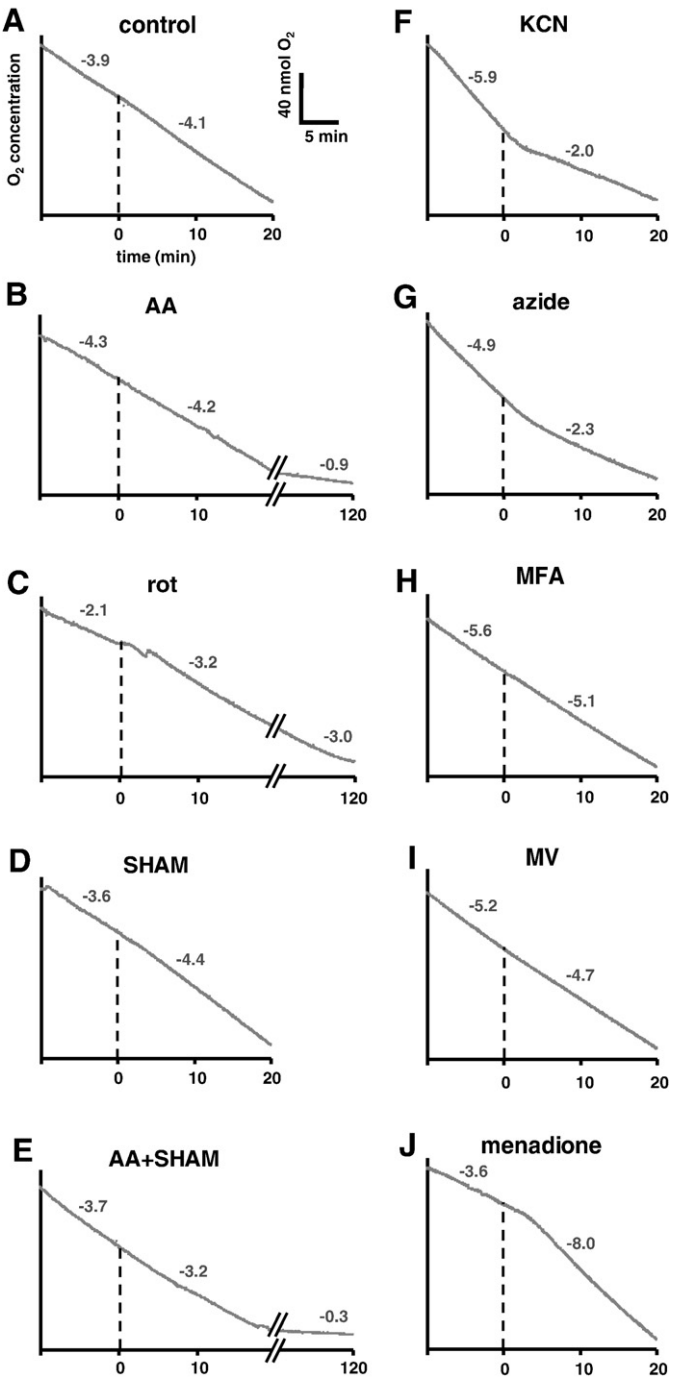

Fig. 2. Impact of mitochondrial inhibitors on oxygen consumption of *Arabidopsis* leaves. Timecourses of oxygen uptake of *Arabidopsis* rosette leaves in response to treatment with mitochondrial inhibitors in the dark. (A) 0.2% ethanol (control); (B) 20 μM antimycin A (AA); (C) rot, 50 μM rotenone; (D) 2 mM salicylhydroxamic acid (SHAM); (E) 20 μM antimycin A, 2 mM salicylhydroxamic acid (AA + SHAM); (F) 500 μM potassium cyanide (KCN); (G) 500 μM sodium azide (azide), (H) 10 mM monofluoracetate (MFA); (I) 100 μM methyl viologen (MV); (J) 100 μM menadione (men). The vertical line indicates the addition of the inhibitor. The experiment was replicated 3 times each with similar results and a typical timecourse is shown. Rate prior to, 10 min after and 2 h after addition of inhibitor are given above the graphs in nmol O_2 min^{-1}.

application caused a strong and immediate increase in oxygen consumption (78 ± 34%, Fig. 2J).

To investigate whether changes in oxygen uptake rate due to respiratory inhibition can be more generally linked to redox perturbation, the effect of mitochondrial inhibitors on mt-roGFP2 redox state was investigated. Excised *Arabidopsis* leaves were perfused with inhibitors for about 10 min. The data show that antimycin A or SHAM had little effect in isolation (Fig. 3A–C). However, when applied together, there was small but steady increase in the fluorescence ratio of roGFP2, indicating a slow oxidation of the probe (Fig. 3D). Treatment with KCN led to a more extensive oxidation of the probe but which was nevertheless slower than that caused by the positive control, menadione (Fig. 3E, F).

These timecourses indicated that oxidation of the probe was not complete after approximately 10 min of measurement and the respiratory measurements also suggested later changes. To investigate the effect of mitochondrial inhibitors at a later timepoint, a change to the experimental system was required. Rather than continuous perfusion and time-lapse measurement, it was necessary to switch to treatment of whole plants and make 'snapshot' roGFP fluorescence measurements of excised leaves at the end of treatment. This was for practical reasons: it is difficult to maintain an immobilised sample for longer time periods and also to allow a greater range of inhibitors to be assessed (by facilitating parallel treatments). Snapshots were taken after 10 min for comparison with timecourse measurements and for 2 h as a point often used for molecular physiological analyses of the effects of these inhibitors. After 10 min, only two treatments, KCN and MFA caused significant increases in mt-roGFP2 oxidation relative to control (Fig. 4A). However, after 2 h, there were also significant increases caused by AA + SHAM, azide and methyl viologen treatments and further increases after KCN and MFA treatments (Fig. 4A). AA and SHAM in isolation, or rotenone did not cause any change in mt-

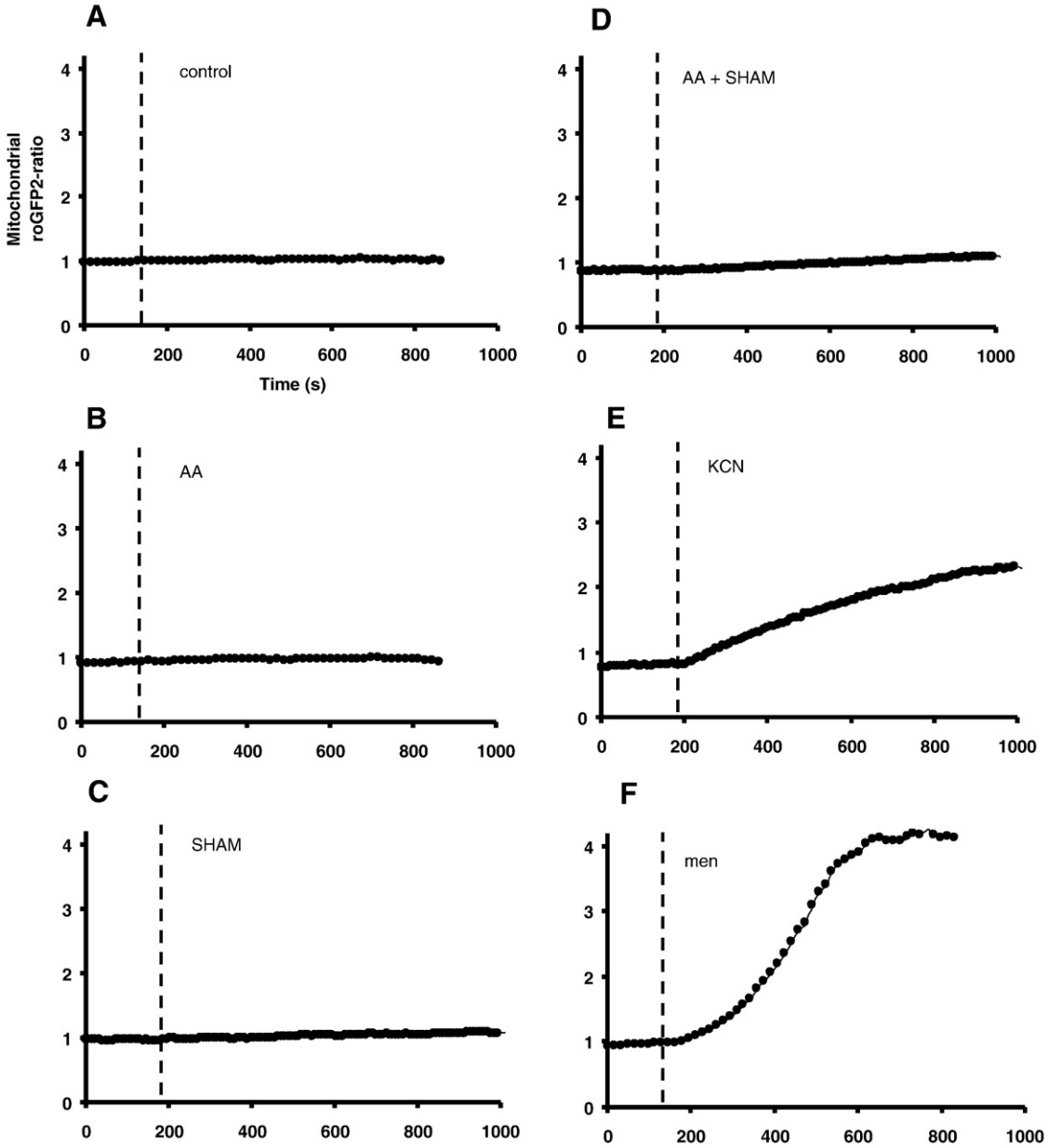

Fig. 3. *In vivo* measurement of mitochondrial roGFP2 oxidation state after treatment of *Arabidopsis* leaves with mitochondrial inhibitors. Fluorescence intensity ratios of mt-roGFP2 after perfusion of excised *Arabidopsis* leaves with different mitochondrial inhibitors in the dark. (A) 0.2% ethanol (control); (B) 20 μM antimycin A (AA); (C) 2 mM salicylhydroxamic acid (SHAM); (D) 20 μM antimycin A + 2 mM salicylhydroxamic acid; (E) 500 μM potassium cyanide (KCN); (F) 100 μM menadione (men). The vertical line indicates the beginning of the treatments. Timecourse perfusion experiments were replicated 3 times with similar results and a typical timecourse is shown.

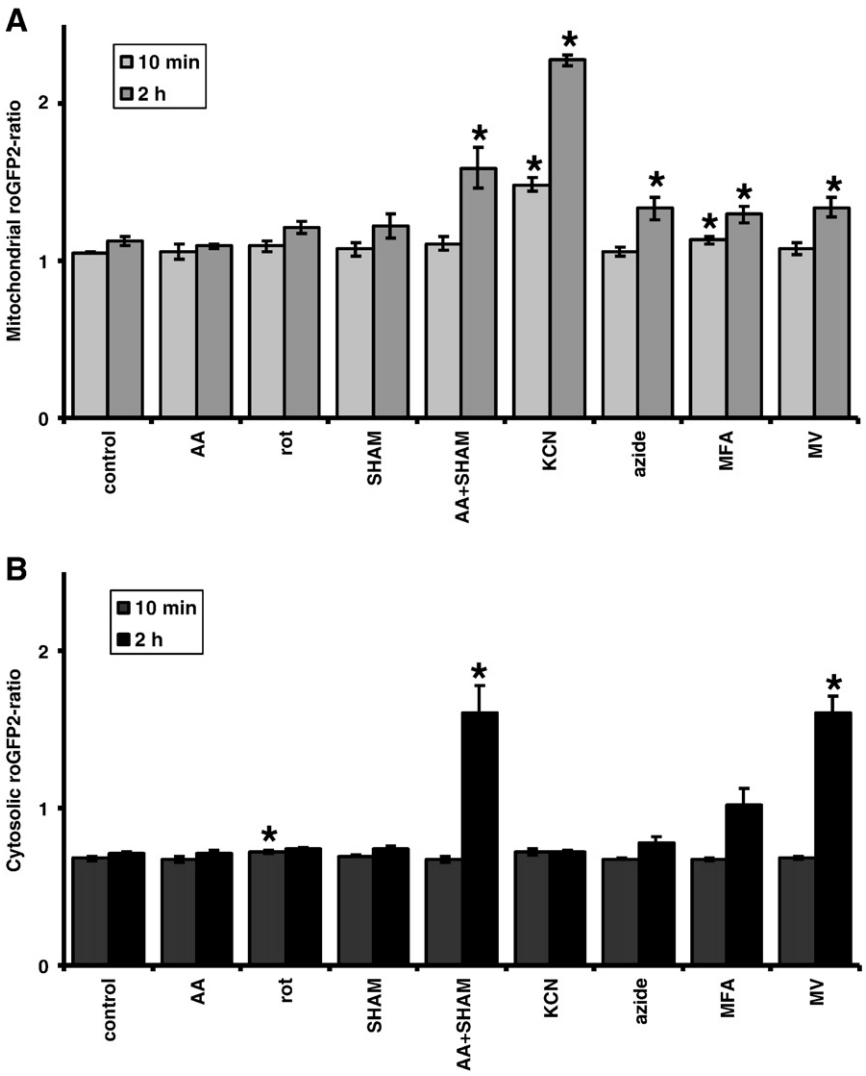

Fig. 4. *In-vivo* measurement of mitochondrial and cytosolic redox state by roGFP2 following treatment of *Arabidopsis* seedlings for 10 min and 2 h with mitochondrial inhibitors. Fluorescence intensity ratios of mt-roGFP2 (A) and c-roGFP2 (B) 10 min and 2 h after treatment of seedlings with different mitochondrial inhibitors in the dark. Control: 0.2% ethanol; AA, 20 μM antimycin A; rot, 50 μM rotenone; SHAM 2 mM salicylhydroxamic acid; AA + SHAM 20 μM antimycin A, 2 mM salicylhydroxamic acid; KCN, 500 μM potassium cyanide; azide, 500 μM sodium azide, MFA, 10 mM monofluoracetate; MV, 100 μM methyl viologen. Data are the mean ± standard error of 5 independent biological replicates. Data were statistically compared to control by *t*-test. $*p<0.05$.

roGFP2 fluorescence ratio. To assess the effect of these treatments on cytosolic roGFP redox state, plants expressing cytosolic roGFP2 were analysed. Two treatments, AA + SHAM and methyl viologen resulted in significant increases in c-roGFP2 oxidation (Fig. 4B). A slight but significant increase in c-roGFP2 fluorescence ratio was observed after 10 min, but not 2 h, of rotenone treatment. However, the change was so small after 10 min as to be considered a statistical artefact.

3.2. Effect of abiotic stress treatment on mitochondrial and cytosolic roGFP oxidation state

Abiotic stress conditions are thought to lead to oxidative stress due to an insufficient capacity to buffer oxidation caused by increased rates of ROS production. Certain stresses such as low temperature and heavy metals are thought to particularly affect mitochondria [27,28]. To assess whether such stresses lead to a perturbation of mitochondrial redox balance, *Arabidopsis* seedlings expressing mt-roGFP2 or c-roGFP2 were exposed to different abiotic stress treatments for 30 min (Fig. 5). High temperature (55 °C), salt stress and exposure to cadmium led to significant increases in mt-roGFP2 oxidation state (Fig. 5A) whereas hyperosmotic stress had no significant impact. Surprisingly, low temperature caused mt-roGFP2 to become slightly, but significantly, more reduced (Fig. 5A). A more detailed examination of the heat stress effect (Fig. 5C) revealed that less severe heat treatments (38 °C and 45 °C) did not cause a significant oxidation of mt-roGFP2. The behaviour of c-roGFP2 to the same stress treatments followed a similar pattern with heat and heavy metal treatments causing significantly increased oxidation, osmotic stress having no effect and low temperature causing a significant decrease in oxidation (Fig. 5B, D). The response of c-roGFP2 to salt stress appeared to be similar to mt-roGFP2, however the response was more variable and the average was not statistically significantly different from control (Fig. 5B).

3.3. Recovery of the redox poise of roGFP in different subcellular compartments following an oxidative challenge

The stress treatments suggest that both mitochondria and cytosol redox state is perturbed to a similar degree by these severe stresses. However, based on degree of protein oxidation, it has been argued that the mitochondrion is more sensitive to redox perturbation than other subcellular compartments [29]. To investigate this further, the rate of

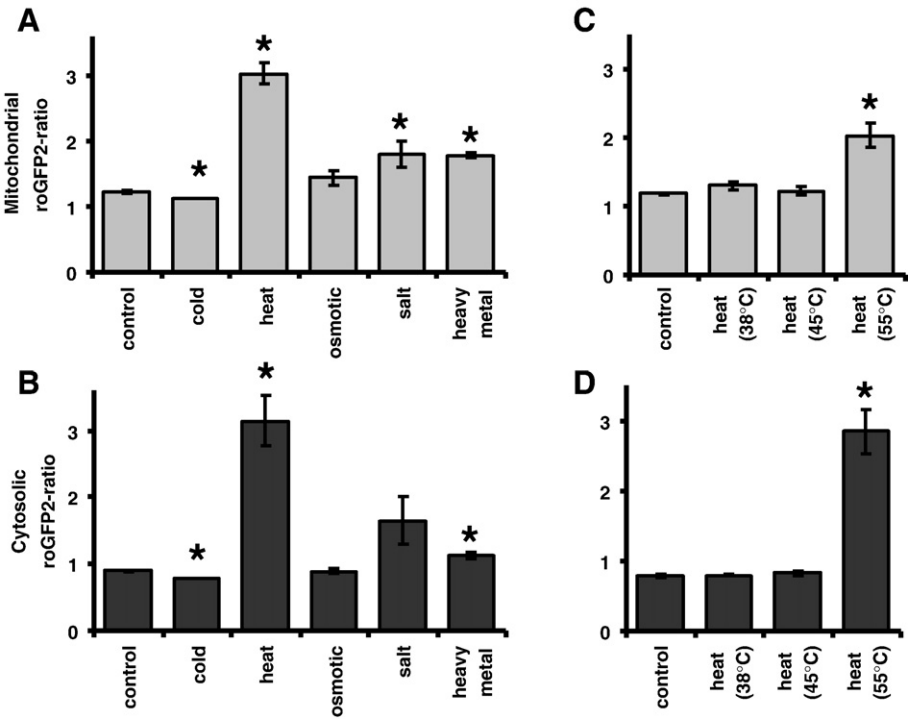

Fig. 5. Measurement of redox changes induced by abiotic stress treatments. Seedlings expressing roGFP2 in the mitochondrion (A, C) and the cytosol (B, D) were treated with various abiotic stresses for 30 min (cold, 0 °C; heat, 55 °C; osmotic, 1 M mannitol; salt, 0.5 M NaCl; heavy metal, 5 mM $CdCl_2$). Heat treatments were further subdivided into 3 different temperatures and applied for 60 min (C, D). Ratio-values were calculated as mean ± standard error. ($n = 5$). Data were statistically compared to control by *t*-test. *$p < 0.05$.

recovery of *Arabidopsis* leaves from oxidative challenge was investigated. Leaves expressing ro-GFP2 in either mitochondria, cytosol or chloroplasts were perfused with 10 mM H_2O_2 (Fig. 6). After approximately 7 min, by which time roGFP2 in all compartments had reached a stable oxidized state, H_2O_2 was removed by perfusion with control medium and the recovery of roGFP redox state monitored. Dramatic differences between the compartments were apparent. roGFP2 in the cytosol showed the fastest recovery with a $t_{0.5}$ of 200 s (±10 s), reaching a value close to the initial resting reduced state within 10 min of removal of H_2O_2. The response in the chloroplast was similar with the roGFP2 recovering to a state only slightly more oxidized than the initial resting value, and a $t_{0.5}$ of 234 s (±20 s). The mitochondria-localised roGFP2 by contrast, did not recover within the monitored time (approximately 30 min). Removal of H_2O_2 resulted in a very slow rate of reduction of mt-roGFP2 such that by the end of the experiment mt-roGFP2 was still approximately 2.5-fold more oxidized than at the resting value at the beginning of the experiment. Note that the different degree of oxidation of roGFP2 by H_2O_2 in the different subcellular locations has been observed before and is not fully understood [18].

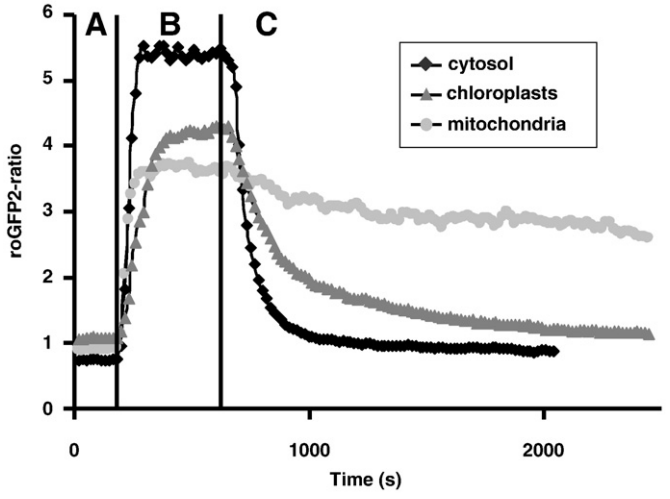

Fig. 6. Recovery of redox poise in different subcellular compartments after oxidative challenge. Leaves expressing roGFP2 in cytosol, chloroplasts and mitochondria respectively were perfused with (A) 0.5× MS medium (0–163 s), (B) 10 mM H_2O_2 in 0.5× MS medium (163–570 s) and again 0.5× MS medium C; (from 570 s onwards). The experiment was repeated 3 times each with similar results. A typical time series experiment is shown.

4. Discussion

Redox-active molecules such as menadione and mitochondrial respiratory inhibitors are frequently used to induce ROS production, e.g. [30], but in most cases the extent to which these treatments perturb the redox state of the mitochondrion is not known. Using mt-roGFP2, we have provided clear evidence that such treatments do induce sufficient ROS to partially oxidize the mitochondrial glutathione pool. Menadione presents by far and away the greatest oxidative challenge, causing nearly complete oxidation of the mt-roGFP. Menadione leads to a rapid increase in oxygen consumption, probably reflecting the strong increase in ROS production.

Mitochondrial respiratory inhibitors caused a much smaller disturbance of mitochondrial redox poise. In fact, rotenone, antimycin A and SHAM which are all known to lead to increased ROS production did not increase oxidation of mt-roGFP2. This may be a result of the flexibility of the plant respiratory chain allowing bypass of the inhibited steps [31]. This explanation is given credence by the fact that antimycin A treatment does lead to mt-roGFP oxidation in the presence of SHAM which prevents bypass of the inhibited complex III by alternative oxidase. Thus, blockage of an electron transport step and its bypass leads to a greater rate of ROS production [32] sufficient to perturb mitochondrial redox state. The implication is that inhibition of a single site of electron transport generates ROS within the normal physiological range that the antioxidant defence machinery can

scavenge without major pertubations of glutathione redox poise. This interpretation is supported by the observed decrease in oxygen consumption which is even more pronounced after 2 h in the presence of antimycin A and SHAM combined than for antimycin A alone.

Given the relatively modest response to classic ROS-generating mitochondrial inhibitors, it is somewhat surprising to observe that inhibitors of complex IV, KCN and azide, led to significant oxidation of mt-roGFP. Even more so when one considers that inhibition of complex IV is not thought to lead to ROS production [33]. Perhaps inhibition of complex IV could cause a 'backing up' of electron transport and result in increased ROS production at complexes II and III or could lead to increased hydrogen peroxide accumulation due to non-specific inhibition of iron-containing redox enzymes (such as peroxidases). In any case a rapid and pronounced inhibition of respiration by these two inhibitors correlates well with the observed redox changes. It was also surprising that application of MFA, which inhibits aconitase, should lead to a significant oxidation of mt-roGFP, although increased ROS production after MFA treatment has been noticed previously [34].

The availability of roGFPs targeted to other subcellular compartments allows the extra-mitochondrial effect of the treatments to be assessed. We used cytosolic roGFP2 to monitor extra-mitochondrial changes. Several of the treatments caused oxidation of c-roGFP2. The most pronounced cytosolic oxidation was generated by menadione. Since no mechanism is known by which menadione can generate ROS directly in the cytosol, this could indicate diffusion of ROS from the organelles. Alternatively, there may be some equilibration between mitochondrial and cytosolic glutathione pools, Antimycin A + SHAM also led to oxidation of the cytosolic roGFP2. This could also be due to diffusion of ROS from the mitochondrion, especially given that inhibition of complex III can lead to generation of ROS in the intermembrane space [35] from which ROS could readily pass through the outer mitochondrial membrane via porin. The effects of KCN, azide and MFA on roGFP oxidation in the mitochondrion were not observed in the cytosol. The fact that it was possible to observe compartment-specific changes in ro-GFP oxidation state strongly suggests that the redox potentials of the different compartments are not in equilibrium. The extent of diffusion of ROS from the mitochondrion may depend on either the amount of ROS production and the exact site of production and this may, in part, explain the differential effect of different inhibitors on cytosolic roGFP oxidation state.

The flexibility of the mitochondrial respiratory chain and the ability to withstand single-respiratory complex inhibition without perturbation of mitochondrial glutathione redox poise raise the question as to how abiotic stress situations affect mitochondrial redox status. A preliminary investigation of a number of abiotic stresses, revealed that severe abiotic stress can lead to oxidation of the mitochondrial roGFP. Further work will be required to establish whether this is true in more physiologically relevant conditions found in the field. For example, while severe heat stress (55 °C) sufficient to induce programmed cell death [36] causes oxidation of mitochondrial roGFP, slightly less severe heat treatments (although still sufficient to induce a heat stress response [37]) had no effect on roGFP redox state.

A significant observation in this context was that mitochondria appear to recover more slowly from an oxidative insult than either the cytosol or chloroplast. There are several possible explanations for this phenomenon. First, it could be a measurement artefact caused by differences in the interactions of roGFP with endogenous glutaredoxins in the different subcellular compartments. roGFP does not interact directly with glutathione but does so via glutaredoxins. It is conceivable that different properties of the glutaredoxins in different compartments could alter the rate of redox equilibration between roGFP and glutathione. However, this is unlikely because the rate of oxidation by roGFP in mitochondria and cytosol after treatment with menadione is similar. It seems more likely that either a reduced capacity of the mitochondria to buffer oxidation, or a persistence of ROS production beyond the end of the treatment could be responsible. The latter may occur due to oxidative damage of mitochondrial electron transport chain complexes leading to greater production of ROS, a feed-forward loop that is a central part of the mitochondrial theory of ageing in mammals [38].

Further investigations will be required to establish the basis of this slower recovery of mitochondrial redox from oxidation. However, given the potential signalling role of glutathione redox state, it raises the intriguing possibility that the mitochondrion could act as sensor of cellular redox perturbation.

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