J Bioenerg Biomembr (2011) 43:237–246
DOI 10.1007/s10863-011-9357-4

Characterization of *Rubus fruticosus* mitochondria and salicylic acid inhibition of reactive oxygen species generation at Complex III/Q cycle: potential implications for hypersensitive response in plants

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Received: 16 November 2010 / Accepted: 4 April 2011 / Published online: 25 May 2011
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Abstract In addition to adenosine triphosphate (ATP) production, mitochondria have been implicated in the regulation of several physiological responses in plants, such as programmed cell death (PCD) activation. Salicylic acid (SA) and reactive oxygen species (ROS) are essential signaling molecules involved in such physiological responses; however, the mechanisms by which they act remain unknown. In non-photosynthesizing tissues, mitochondria appear to serve as the main source of ROS generation. Evidence suggests that SA and ROS could regulate plant PCD through a synergistic mechanism that involves mitochondria. Herein, we isolate and characterize the mitochondria from non-photosynthesizing cell suspension cultures of *Rubus fruticosus*. Furthermore, we assess the primary site of ROS generation and the effects of SA on isolated organelles. Mitochondrial Complex III was found to be the major source of ROS generation in this model. In addition, we discovered that SA inhibits the electron transport chain by inactivating the semiquinone radical during the Q cycle. Computational analyses confirmed the experimental data, and a mechanism for this action is proposed.

Keywords Mitochondria · Salicylic acid · Reactive oxygen species · *Rubus fruticosus*

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Introduction

The major function of the mitochondria in animals and plants is the production of adenosine triphosphate (ATP), which occurs via interaction of metabolic pathways with electron transport through mitochondrial respiratory chain (MRC) complexes I–IV in the inner organelle membrane. In addition to the classical MRC complexes I–IV, plant mitochondria are comprised of the following: alternative NAD(P)H dehydrogenases (inner and outer) that donate electrons directly to ubiquinone, alternative oxidase (AOX) that receives electrons from ubiquinone and directly donates them to oxygen to circumvent the cytochrome pathway, and the plant uncoupling protein (PUMP) that distributes protons in the mitochondrial matrix to evade F_oF_1-ATP synthase (Juszczuk and Rychter 2003; Rasmusson et al. 2004; Vercesi et al. 2006). However, unlike the classical MRC complexes, these alternative enzymes do not generate proton pumping.

In addition to ATP production, mitochondria have been implicated in the regulation of several physiological

responses. Similar to animals, plant mitochondria integrate diverse stress signals, and evidence suggests that they could initiate the programmed cell death (PCD) execution pathway (Lam et al. 2001). Plant PCD appears to be involved in tracheary element formation (Fukuda 2004), organogenesis (Bozhkov et al. 2005), hypersensitive response (Mur et al. 2008) and other stresses (Lam 2008). Reactive oxygen species (ROS) generation by mitochondria may be a common factor by which PCD is activated both in animals and plants (Blackstone and Green 1999).

Mitochondria produce ROS when molecular oxygen is partially reduced by the electrons that leak from the electron transport chain (ETC). Superoxide is the primary ROS produced in this circumstance. However, the major ROS released from the mitochondria is membrane-permeable hydrogen peroxide (H_2O_2), which results from dismutation of the superoxide anion either spontaneously or catalyzed by superoxide dismutase (Turrens 2003; Tahara et al. 2009). In animals, ROS are generated mainly by mitochondrial Complexes I and III (Turrens and Boveris 1980; Turrens et al. 1982; Barja 1999; McLennan and Degli Esposti 2000; Kushnareva et al. 2002; Tahara et al. 2009). In Complex I, ROS are produced through the forward transfer of electrons derived from NADH and the reverse transfer of electrons derived from succinate (Turrens 2003; Tahara et al. 2009). In the Complex III, ROS are generated through the formation of the radical ubisemiquinone (UQ^{-}•), which is an intermediate in the Q cycle (Trumpower 1990). Additionally, the MRC inhibitor, antimycin-A, increases the steady-state levels of UQ^{-}• by binding to Complex III, thereby increasing superoxide formation. In plants, the mechanism of ROS generation is more complex due to the contribution of chloroplasts and alternative enzymes, present in mitochondria (Møller 2001). Thus, few models are available for studies.

Salicylic acid (SA) is a phenolic compound that is involved in plant signaling. Unlike animals, plants can synthesize SA and activate SA-dependent physiological responses (Klessig and Malamy 1994). A key role of SA in plants is the activation of a special type of PCD, hypersensitive response (HR), which is related to cell defense (Alvarez 2000; Mur et al. 2008). HR is locally triggered and restricts pathogens at the infection site, avoiding their spreading (Morel and Dangl 1997). This local response has been associated with the activation of the systemic acquired response (SAR), which confers resistance to the other plant regions beyond the site of infection (Ryals et al. 1996). The spatiotemporal pattern of SA accumulation in HR suggests that it contributes to the controlled timing and extent of cell death. Although SA alone does not produce hypersensitive cell death, it may play an indirect but essential role in HR by regulating cell death mechanisms (Mur et al. 2008). SA may rapidly accumulate in cells at the HR site (Bi et al. 1995); when externally applied at millimolar concentrations (Maxwell et al. 2002; Raskin et al. 1987) its accumulation is increased by approximately 1000-fold (Norman et al. 2004).

Within the isolated mitochondria from tobacco cells, SA has been reported to induce the uncoupling and inhibition of MRC, which consequently modifies the alternative oxidase expression and cell ATP levels (Xie and Chen 1999; Norman et al. 2004). This evidence suggests that this signaling involves mitochondria. Moreover, both SA accumulation and ROS generation regulate physiological responses in plants (Shirasu et al. 1997; Klessig et al. 2000). In the present work we isolate and characterize the mitochondria from cell suspension cultures of *Rubus fruticosus* (*R. fructicosus*, blackberry). We also assess the primary ROS generation site, as well as the effects of SA on this model. Evidence supports that MRC Complex III is the major ROS generation site in *R. fruticosus* and that SA inhibits the ETC by inactivating the semiquinone radical during the Q cycle. A mechanism based on a computational study is proposed.

Material and methods

Chemicals

Salicylic acid (SA) was purchased from Sigma and prepared in water; the pH was adjusted to 6.5 with KOH (Xie and Chen 1999). All other chemicals were of the highest grade (Sigma, St. Louis, MO, USA).

Plant material

R. fruticosus L. suspended cells, originally derived from cambial explants of twigs, were grown as previously described (Hustache et al. 1975). The culture medium contained the following macroelements: 56 mg/ml $CaCl_2$, 750 mg/ml KCl, 121 mg/ml $MgSO_4$, 600 mg/ml $NaNO_3$ and 109 mg/ml KH_2PO_4. The medium also contained the following microelements: 0.054 mg/ml $AlCl_3$, 0.030 mg/ml $CuSO_4$, 1 mg/ml H_3BO_3, 0.01 mg/ml KI, 0.075 mg/ml $MnSO_4$, 0.03 mg/ml $NiCl_2$ and 1 mg/ml $ZnSO_4$. The medium was supplemented with 50 g/l sucrose, 1 mg/ml thiamine and 1 mg/ml $FeCl_3$. Cells were subcultured after 6 weeks.

Mitochondria isolation

Mitochondria were isolated by conventional differential centrifugation as previously described, with modifications, for cell suspension cultures of *Araucaria angustifolia*

(Mariano et al. 2004). Cells in exponential growth (17–21 days) were filtered using a membrane with a 10 μm cut-off and then smoothly homogenized in a Potter-Elvehjem homogenizer in the presence of a cold extraction medium containing 250 mM sucrose, 10 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)-KOH (HEPES-KOH), 2 mM ethylene glycol bis (2-aminoethyl ether)-N, N,N′,N′-tetraacetic acid (EGTA), 3 mM cysteine and 0.2 g % fatty acids free-bovine serum albumin (BSA) (pH 7.6, 30 g of cells/100 ml of medium). The homogenate was centrifuged at 1,000 × *g* for 10 min. The supernatant was then centrifuged at 15,000 × *g* for 10 min, and the resulting pellet was resuspended in a cold wash medium containing 250 mM sucrose, 10 mM HEPES-KOH, 0.25 mM EGTA and 0.2 g% BSA (pH 7.2). This fraction was then transferred to a single tube and centrifuged at 1,000 × *g* for 10 min. The resulting supernatant was centrifuged at 15,000 × *g* for 10 min. The pellet (mitochondrial fraction) was resuspended in 1 ml of the washing medium and held on ice until further use. The protein content was estimated according to Lowry et al. (1951).

Oxygen consumption assay

Oxygen consumption was monitored to assess mitochondrial respiration rate (state 3 and state 4) with a Clark-type electrode, connected to a Gilson oxygraph, in a standard reaction medium containing 250 mM sucrose, 2 mM KCl, 10 mM HEPES-KOH pH 7.2, 0.2 g% BSA fatty acids-free, 2 mM Pi, 1 μM GTP, 0.5 mM EGTA, 5 mM $MgCl_2$ and 1.0 mg of mitochondrial protein. The final volume amounted to 1.8 ml; all measurements were made at 28 °C.

Mitochondrial membrane potential ($\Delta\psi$) assay

The $\Delta\psi$ was monitored fluorometrically in a Hitachi F-4500 spectrofluorometer at 495 nm (excitation) and 586 nm (emission) wavelengths and with a 5-nm slit width (Petrussa et al. 1992). The incubation medium consisted of the reaction medium, which was supplemented with 5 μM rotenone, 5 μM safranine *O* and 1.0 mg of mitochondrial protein (final volume of 2 ml, 28 °C). Measurements were initiated with 5 mM succinate or 2 mM NADH. To verify the $\Delta\psi$ dissipation, 1 μM of carbonyl-cyanide-*p*-trifluorometoxyphenyl-hydrazone (FCCP) was added. The mitochondrial oxidative phosphorylation was assessed by additions of ADP (200 μM) and oligomycin (2.0 $\mu g.ml^{-1}$). The presence of uncoupling protein (PUMP) was examined in the absence of BSA and GTP, which were further added (0.2 g% BSA and 1 μM GTP). The dye safranine *O* is electrophoretically distributed into the mitochondria and shifts the fluorescence in a manner that is inversely proportional to the generated $\Delta\psi$. Therefore, data can be expressed in relative fluorescence units (RFU) or as $\Delta\psi$ (mV), which is obtained from a calibration curve using a K^+ gradient and the Nernst equation, as previously described (Akerman and Wikström 1976).

Mitochondrial ROS assay

The mitochondrial ROS levels were monitored by observing the fluorescence produced after the oxidation of fluorescent probes, 2-7-dichlorodihydrofluorescein diacetate (H_2DCFDA) and Amplex Red. H_2DCFDA primarily detects H_2O_2 (McLennan and Degli Esposti 2000). Mitochondria (1.0 mg of protein) were incubated with 5 μM H_2DCFDA in 2 ml of the reaction medium at 28 °C, and 5 mM succinate was added to initiate mitochondrial respiration. H_2DCFDA is degraded by mitochondrial esterases, releasing DCFDA, which can be oxidized to DCF (2-7-dichlorodihydrofluorescein) by ROS. DCF fluorescence was monitored continuously by a Hitachi F-4500 spectrofluorometer at 503 nm (excitation) and 529 nm (emission) and with a 5-nm slit width. The Amplex Red (25 μM) oxidation was monitored in the presence of 0.5 U/ml horseradish peroxidase (HRP), using succinate (5 mM) as respiratory substrate. Amplex Red is oxidized in the presence of extramitochondrial HRP bound to H_2O_2, generating resorufin, which was monitored at 563 nm (excitation) and 587 nm (emission). Antimycin A (2.0 μg/ml), rotenone (2 μM), myxothiazol (2 μM), KCN (1 mM) and SA (0.25–5.0 mM) were incubated with the mitochondria before data collection. We could not use KCN or SA in the Amplex red assay because they inhibit HRP (Møller 2001; Alvarez 2000). Control assays were performed in the absence of mitochondria.

MRC Complex II and II+III activities assay

Complex II (succinate: ubiquinone reductase) activity was measured spectrophotometrically, following the decrease in absorbance resulting from the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm, as previously described (Barrientos 2002). Complex II+III (succinate cytochrome *c* reductase) activity was measured following the increase in absorbance resulting from the reduction of exogenously added cytochrome *c* at 550 nm (Barrientos 2002). Malonate (10 mM), an inhibitor of Complex II, was used as a control. A mitochondrial suspension (50 μg/ml) was incubated with SA in the specific reaction medium for Complex II (10 mM KH_2PO_4, pH 7.8, 2 mM EDTA, 1 mg/ml BSA, 4 μM rotenone and 0.2 mM ATP) or for Complex II+III (10 mM KH_2PO_4, pH 7.8, 2 mM EDTA, 1 mg/ml BSA, 240 μM KCN, 4 μM rotenone and 0.2 mM ATP). The presence of

SA was tested in the absence of mitochondria to exclude any interference. The activities of complexes were expressed as R, which represents the absorbance obtained for the control (R=1.0) in relation to the absorbance obtained for treated samples.

Computational studies

All subsystems were optimized by the B3LYP/6-31+G(d,p) model (Becke 1993; Lee et al. 1988; Hehre et al. 1972; Hariharan et al. 1974) using Gaussian 03 suite programs (Frisch et al. 2004). A minimum value for potential surface energy was characterized by analysis of the harmonic vibrational frequencies that presented only positive values. The influence of the solvent system (physiological pH) was incorporated by using the Polarizability Continuous Model (PCM) (Mertius and Tomasi 1982). The electron affinity (EA) was computed as the Gibbs energy difference between the radical anion and neutral molecule.

Statistical analysis

Data are represented as averages ± SEM of 5–10 repetitions using distinct preparations. Multiple comparisons were performed utilizing one-way ANOVA, followed by Tukey or Dunnetts' post-test. The comparisons were conducted using GraphPad Prism software.

Results

Functional characterization of *R. fruticosus* mitochondria

The isolation of mitochondria from cell suspension cultures of *R. fruticosus* (blackberry) was performed as previously described (Mariano et al. 2004; Petrussa et al. 2008). We did not employ a Percoll gradient, commonly used to remove plastids and peroxisomes, due to its drastic reduction of mitochondrial protein. In fact, *R. fruticosus* cells are non-photosynthetic cells, and contamination by plastids is unlikely to occur. Because contamination by peroxisomes was also low (catalase activity of 0.50 μmol O_2. $min^{-1}.mg^{-1}$), we chose to work with the crude mitochondrial preparation.

To characterize the *R. fruticosus* mitochondria, state 3 and state 4 mitochondrial respiration rates, corresponding respectively to ADP-stimulated and basal respirations, as well as the respiratory control ratio (RCR, state 3/state 4), were assessed in the presence of different respiratory substrates. The mitochondria oxidized NAD^+- (a cocktail containing malate/glutamate/pyruvate) and FAD-linked succinate substrates (Table 1); the highest state 3 respiration rate was achieved with the NAD^+-linked substrate mixture.

Table 1 Respiration rates of mitochondria isolated from cell suspension cultures of *R. fruticosus* respiring with different substrates

Respiratory substrates	State 3 rate	State 4 rate	RCR
NADH	113.3±35.0	61.6±11.5	1.82±0.32
NADH/Flavone	43.3±7.6	38.3±10.4	1.13±0.15
NADH/Ca^{2+}	101.6±7.6	63.3±2.8	1.60±0.15
NADPH	60.0±17.3	33.3±10.4	1.81±0.17
NADPH/Ca^{2+}	98.3±2.8	60.0±8.6	1.65±0.19
Glu/Mal/Pyr	151.6±28.8	101.6±37.5	1.57±0.23
Succ/Rot	68.3±10.4	51.6±10.4	1.34±0.15

The experimental conditions are previously described. The reaction medium contained 250 mM sucrose, 2 mM KCl, 10 mM HEPES-KOH pH 7.2, 0.2 g% BSA fatty acids-free, 2 mM Pi, 1 μM GTP, 0.5 mM EGTA, 5 mM $MgCl_2$ and 1 mg/ml of mitochondrial protein (1.8-ml final volume, 28 °C). State 3 respiration was initiated with 200 μM ADP. The concentrations used were the following: 2 mM NAD(P)H, 1 mM Ca^{2+}, 0.5 mM flavone, 5 mM glutamate/malate/pyruvate (Glu/Mal/Pyr), 5 mM succinate (Succ) and 2 μM rotenone (Rot). The respiration rate is given as ng atoms O. min^{-1}. mg^{-1} of mitochondrial protein ± S.D. values of three independent experiments

RCR Respiratory Control Ratio

In addition, exogenously added NADH or NADPH induced the respiration of *R. fruticosus* mitochondria, suggesting the presence of external NAD(P)H dehydrogenase. Flavone, a classical inhibitor of alternative NADH dehydrogenase, decreased the respiration rate of NADH-respiring mitochondria by 40%. In contrast, Ca^{2+} (1 mM) increased the respiration rate supported by NADPH, suggesting an affinity of external NADPH dehydrogenase for the cation. On the other hand, Ca^{2+} did not significantly increase respiration of the mitochondria exposed to exogenous NADH.

Figure 1 reports the respiration rates of *R. fruticosus* mitochondria, supported by NADH (Fig. 1a) or succinate (Fig. 1b). The uncoupler, carbonyl-cyanide-*p*-trifluorometoxyphenyl-hydrazone (FCCP), greatly increased both respiration rates, indicating that organelles became well-coupled. In addition, salicylhydroxamic acid (SHAM) inhibited this stimulated respiration, suggesting the presence of an alternative oxidase (AOX). Only a complete inhibition was achieved in the presence of both SHAM and KCN; therefore, the presence of the cyanide-resistant pathway is also suggested.

Mitochondrial membrane potential ($\Delta\psi$) is a very sensitive indicator of the energy-coupling condition of mitochondria. The electron flow through MRC is coupled to the proton pumping from the mitochondrial matrix to the intermembrane space. These actions generate a proton-motive force, comprised of a chemical and electrical potential energy ($\Delta\psi$) that drives ATP synthesis by F_oF_1-ATP synthase. The formation of $\Delta\psi$, after succinate

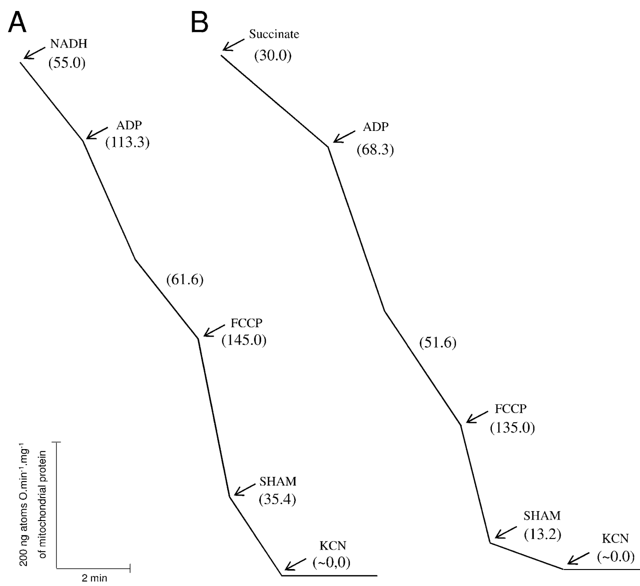

Fig. 1 Representative tracings (from 5 independent experiments) for the respiration of mitochondria isolated from cell suspension cultures of *R. fruticosus*. The reaction medium contained 250 mM sucrose, 2 mM KCl, 10 mM HEPES-KOH pH 7.2, 0.2 g% BSA fatty acids-free, 2 mM Pi, 1 μM GTP, 0.5 mM EGTA, 5 mM $MgCl_2$ and 1 mg/ml of mitochondrial protein (1.8-ml final volume, 28 °C). Respiratory substrates: **a** 2 mM NADH and **b** 5 mM Succinate. FCCP (1 μM), SHAM (1 mM), KCN (1 mM) and ADP (200 μM) were added where indicated by arrows. The rates of oxygen consumption are presented in ng atoms of O. min^{-1}. mg^{-1} of mitochondrial protein (*numbers in squares*)

addition to *R. fruticosus* mitochondria, denotes inner mitochondrial membrane integrity, and its dissipation by FCCP denotes that mitochondria became well-coupled (Fig. 2a). The oxidative phosphorylation capacity is demonstrated by the responses of the mitochondria to ADP and the ATP synthase inhibitor, oligomycin. ADP addition was followed by a small upward deflection, compatible with the utilization of $\Delta\psi$ to drive ATP phosphorylation (Akerman and Wikström 1976); oligomycin fully reversed this effect (Fig. 2b).

To assess the uncoupling protein (PUMP) within *R. fruticosus* mitochondria, the $\Delta\psi$ was monitored both in the absence or presence of GTP and BSA; GTP is a direct inhibitor of PUMP while BSA chelates the fatty acids that activate PUMP (Vercesi et al. 2006). $\Delta\psi$ generation by succinate-respiring mitochondria was found to be lower in the absence (Fig. 2c), rather than in the presence (Fig. 2a), of BSA. This is consistent with prior evidence; due to the presence of PUMP, a well-coupled state of plant mitochondria is achieved only with BSA (Vercesi et al. 2006). Moreover, addition of GTP with BSA increased the $\Delta\psi$ at the same level of the mitochondria respiring with succinate, in the presence of both BSA and GTP (Fig. 2a).

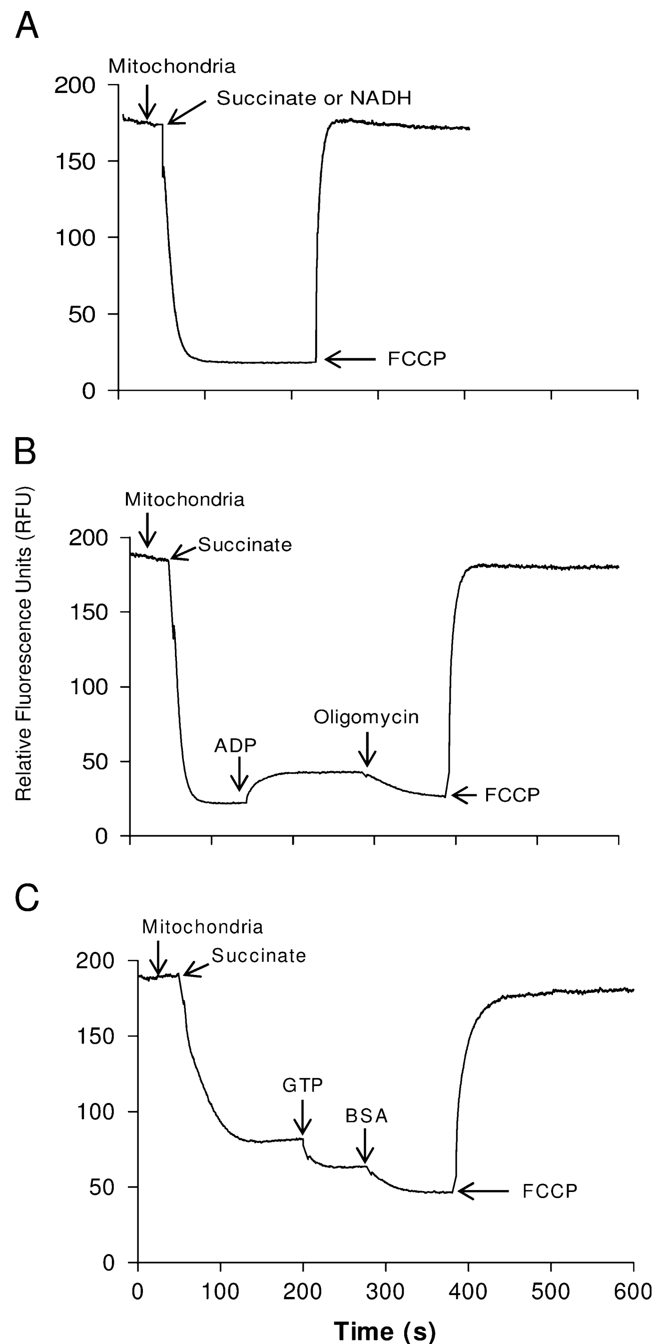

Fig. 2 Representative tracings (from 5 independent experiments) for the $\Delta\psi$ of mitochondria isolated from cell suspension cultures of *R. fruticosus*. The same conditions described in Fig. 1 were used, except that the medium (2-ml final volume) was supplemented with 5 μM rotenone and 5 μM safranine *O*. **a** Mitochondria respiring with 5 mM succinate or 2 mM NADH, followed by 1 μM FCCP-induced $\Delta\psi$ dissipation is shown. **b** Mitochondria respiring with succinate, followed by 200 μM ADP and 2.0 μg/ml oligomycin additions to verify oxidative phosphorylation are shown. **c** Mitochondria respiring with succinate, followed by 1 μM GTP and 0.2% BSA to verify the presence of PUMP is shown (for this specific assay, the initial incubation medium did not include GTP and BSA)

Mitochondrial ROS generation by *R. fruticosus* mitochondria

In plants, ROS are generated by chloroplasts, peroxisomes and mitochondria; in non-photosynthesizing tissues, the mitochondrial generation of ROS prevails (Møller 2001). MRC Complexes I and III are the primary sites of ROS generation in animals (Turrens 2003). In Complex I, both forward and reverse electron transfers occur. The former involves the electrons derived from NADH, which may generate superoxide at the FMN and Q binding sites (Lambert and Brand 2004). The latter involves the flow of electrons derived from succinate to Complex I (Turrens 2003; Tahara et al. 2009). The complex I inhibitor rotenone usually prevents superoxide generation in succinate-respiring mitochondria (Tahara et al. 2009).

In the Complex III, ROS is generated from the autoxidation of the semiquinone radical (Q^{-}•), formed during the Q cycle (Trumpower 1990). Coenzyme Q is fully reduced (QH_2, ubiquinol) in the inner side of the inner mitochondrial membrane (Q_i site) and then migrates to the outer side (Q_o site) to donate two electrons. The first electron is donated to cytochrome c_1 via the Rieske Fe-S protein to form Q^{-}•, and the second electron is donated from this radical species to cytochrome *b*. Eventually, some of the electrons leak to oxygen, generating superoxide. Q^{-}• may also be formed at the Q_i site, and therefore, superoxide can be generated at both Q_o and Q_i sites (Cape et al. 2006). The Complex III inhibitor, antimycin-A (AA), increases the steady-state level of Q^{-}• by binding to cytochrome *b*, increasing the probability of oxygen to access this radical and generate superoxide (Turrens 2003). If the Rieske Fe-S protein is inhibited by myxothiazol, KCN or bycytochrome *c* depletion, then the electrons are prevented from reaching molecular oxygen. In these conditions, the increase of superoxide generation is inhibited by antimycin-A (Turrens et al. 1985).

The ROS generated by the mitochondria isolated from *R. fruticosus* was assessed under the same experimental conditions employed for mitochondrial respiration and $\Delta\psi$ studies. The most probable ROS generation site was investigated in succinate-respiring mitochondria; ROS was monitored by means of both H_2DCFDA (Fig. 3a, b) and Amplex red (Fig. 3c) assays. While interference of the MRC inhibitors used in our studies with the H_2DCFDA assay is negligible (McLennan and Degli Esposti 2000), the HRP used in the Amplex red assay is inhibited by KCN (Møller 2001). Therefore, the effect of this inhibitor was not examined by this assay.

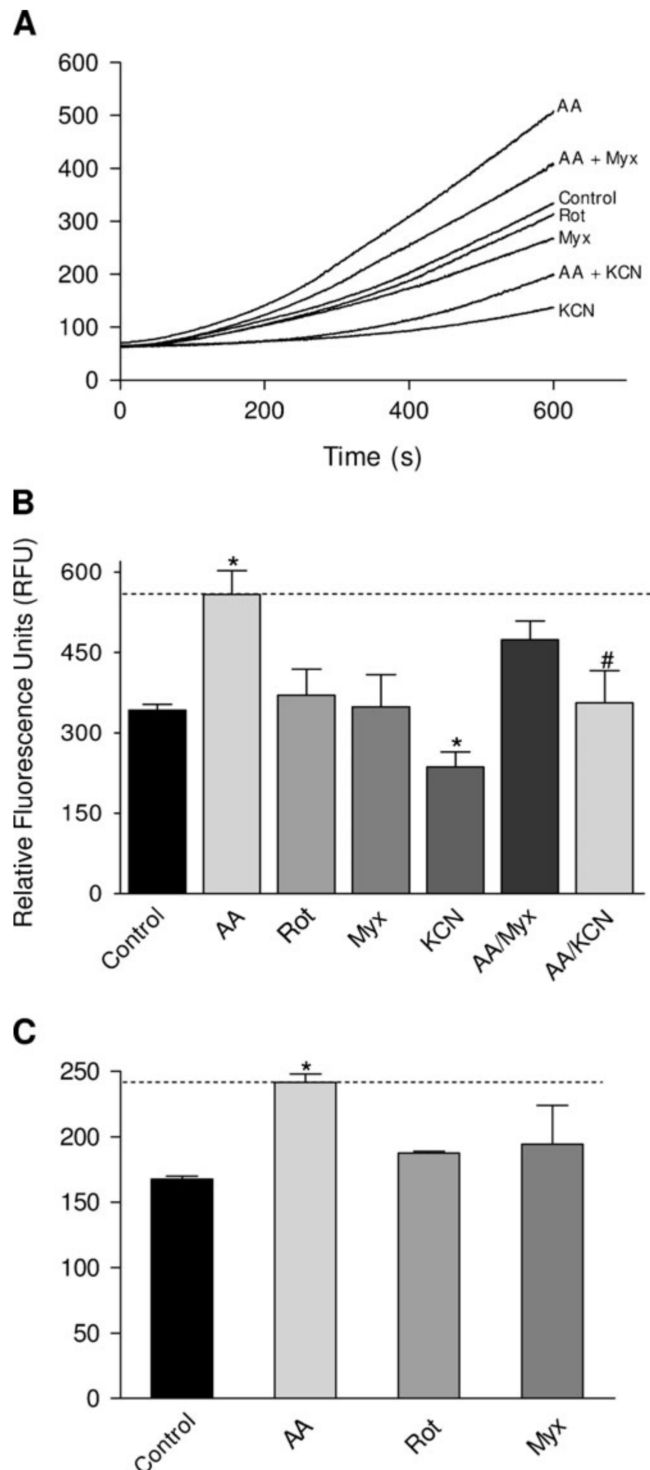

Fig. 3 ROS generation in succinate-respiring mitochondria isolated from cell suspension cultures of *R. fruticosus*. Mitochondria (1.0 mg of protein) were incubated in 2.0 ml of the reaction medium described in Fig. 1 and were supplemented with 5 mM succinate at 28 °C. **a** Representative tracings (from 5 independent experiments) monitored by the H_2DCFDA (5 μM) assay, as previously described. Antimycin-A (AA, 2.0 μg/ml), rotenone (Rot, 2 μM), myxothiazol (Myx, 2 μM) and KCN (1 mM) were incubated with the mitochondria prior to experimentation. **b** Means ± S.D. of ROS generation as measured in **a**, after 600 s incubation, is shown. **c** Means ± S.D. of ROS generation assessed by the Amplex red assay, after 600 s incubation, is shown; the reaction medium was supplemented with 25 μM Amplex red and 0.5 U/ml HRP. For **b** and **c**: *$p<0.001$ vs. control and # $p<0.001$ AA/KCN vs. AA were calculated

In agreement with animal mitochondria, the basal ROS generation in succinate-respiring *R. fruticosus* mitochondria was stimulated by the mitochondrial Complex III inhibitor, AA, as shown in Fig. 3 (McLennan and Degli Esposti 2000; Turrens 2003; Tahara et al. 2009). The MRC inhibitors, rotenone and myxothiazol, did not significantly affect basal ROS levels. However, KCN, a specific Complex IV inhibitor, partially inhibited both basal and AA-induced ROS generation (Fig. 3a). Rotenone did not cause any effect on ROS generation; thus, we excluded any influence of the reverse electron transfer (Tahara et al. 2009). Therefore, our results suggest that ROS generation in succinate-respiring mitochondria isolated from *R. fruticosus* occurs mainly at Complex III. Also, myxothiazol, either alone or in the presence of AA, did not affect mitochondrial ROS generation (Fig. 3). As this inhibitor decreases ROS generation at Complex III by preventing Q^{-}• formation, our results suggest that ROS generation at Complex III occurs through another site beyond Q_o, as it has been recently proposed to occur in animal mitochondria (Dröse and Brandt 2008).

Effects of SA on *R. fruticosus* mitochondria

The effects of SA on *R. fruticosus* mitochondria that are respiring with succinate was assessed with regard to the mitochondrial respiration rate of state 3, the $\Delta\psi$ dissipation and ROS generation (Fig. 4). SA (0.5–5.0 mM) did not significantly stimulate the state 4 respiration rate (data not shown), indicating that it does not act as a mitochondrial uncoupler under our conditions. However, SA significantly inhibited the state 3 respiration rate, and maximal inhibition was achieved between 1.0 and 2.5 mM (Fig. 4a). In an apparent association with the inhibition of state 3 respiration, SA dissipated the $\Delta\psi$ in a concentration-dependent manner; a dissipation equivalent to the uncoupler, FCCP, was achieved at 2.5 mM SA (Fig. 4b). For ROS detection, only the H_2DCFDA assay was used, because SA inhibits the HRP used in the Amplex red assay (Durner and Klessig 1995; Alvarez 2000). While inhibition of basal ROS generation by SA (0.25–2.5 mM) was not significant, an inhibition of 40% in AA-stimulated ROS generation was achieved when SA was applied at 2.5 mM (Fig. 4c). When antimycin-A binds to Complex III, the steady-state concentration of Q^{-}• is increased, which in turn increases ROS generation at this level. Therefore, our results suggest that SA inhibits ROS generation by preventing Q^{-}• accumulation during the Q cycle.

Effects of SA on MRC activities

Next, the effects of SA on *R. fruticosus* mitochondria MRC Complex II (succinate: ubiquinone reductase) and Complex II+III (succinate cytochrome *c* reductase) activities were assessed with succinate as the respiratory substrate. The

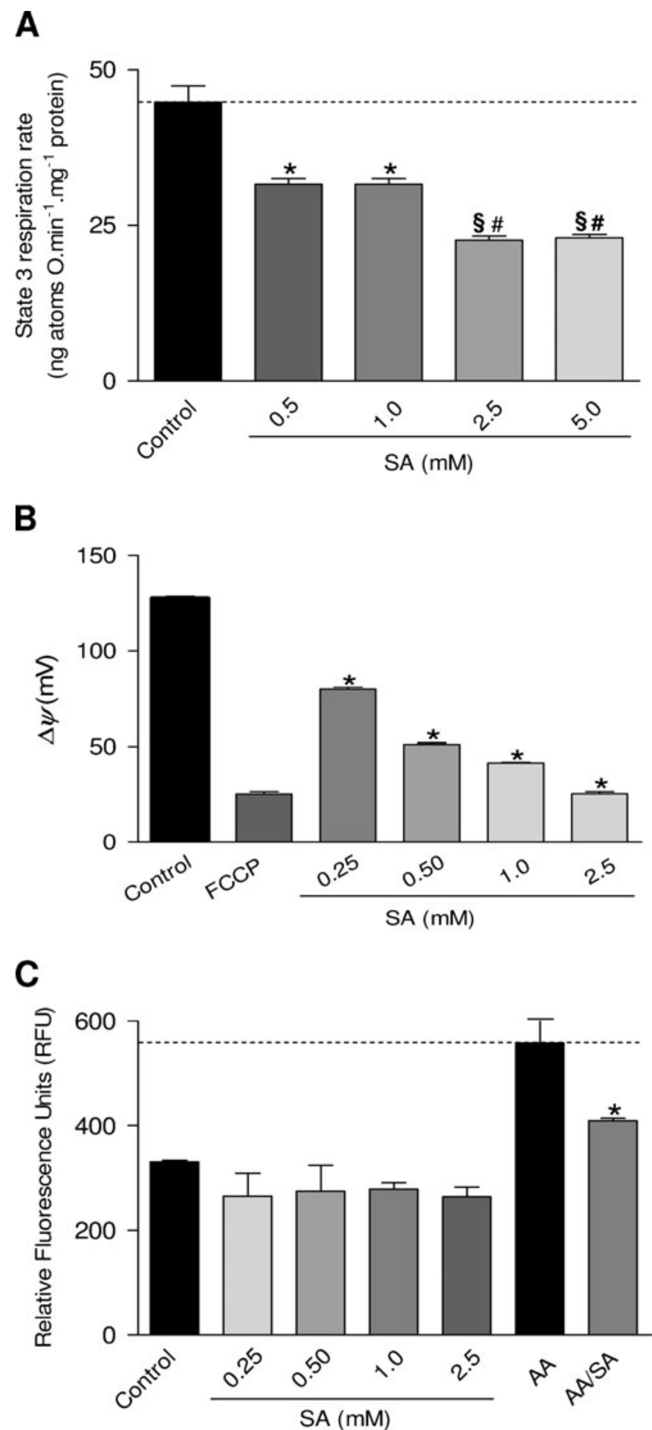

Fig. 4 Effects of SA on succinate-respiring mitochondria isolated from cell suspension cultures of *R. fruticosus*. For all parameters tested, assays were performed as previously described. **a** State 3 respiration rate: *$p<0.01$ vs. control, §$p<0.001$ vs. control, # $p<0.001$ vs. SA 0.5 or 1.0 mM are reported. **b** $\Delta\psi$: *$p<0.001$ vs. control (FCCP, 1 µM) is shown. **c** Basal or AA-induced (2.0 µg/ml) ROS generation, assessed by the H_2DCFDA assay: *$p<0.001$ AA + SA vs. AA is displayed. At least 5 independent experiments were performed

effect of SA on MRC Complex II, that catalyzes the transfer of electrons from succinate to ubiquinone (Davis and Hatefi 1971), was assessed by measuring the reduction of DCPIP, used as an electron acceptor (it was not reduced in the presence of SA alone, data not shown). The reaction was initiated with the acceptor, decylubiquinone, which stimulated the activity of the complex incubated with DCPIP and succinate. Malonate, a Complex II inhibitor, inhibited this activity by 95%, and SA (0.25 and 2.5 mM) did not cause any inhibition of the Complex II activity (Fig. 5a). The transfer of electrons from Complex II to Complex III was assessed by measuring the activity of succinate cytochrome *c* reductase, monitored by the reduction of exogenously added, oxidized cytochrome *c* (Barrientos 2002). SA (2.5 mM) inhibited this activity by 30% (Fig. 5b), whereas, the control, malonate, inhibited it by 95%. A control was performed to verify if SA could reduce cytochrome *c* in the absence of mitochondria, and it was confirmed that SA alone does not affect the redox state of cytochrome *c* (data not shown). The inhibition of succinate cytochrome *c* reductase by SA was low and reached a saturation level, suggesting that SA does not bind covalently to Complex III. These findings correspond with the above results concerning the respiratory parameters of *R. fruticosus* mitochondria.

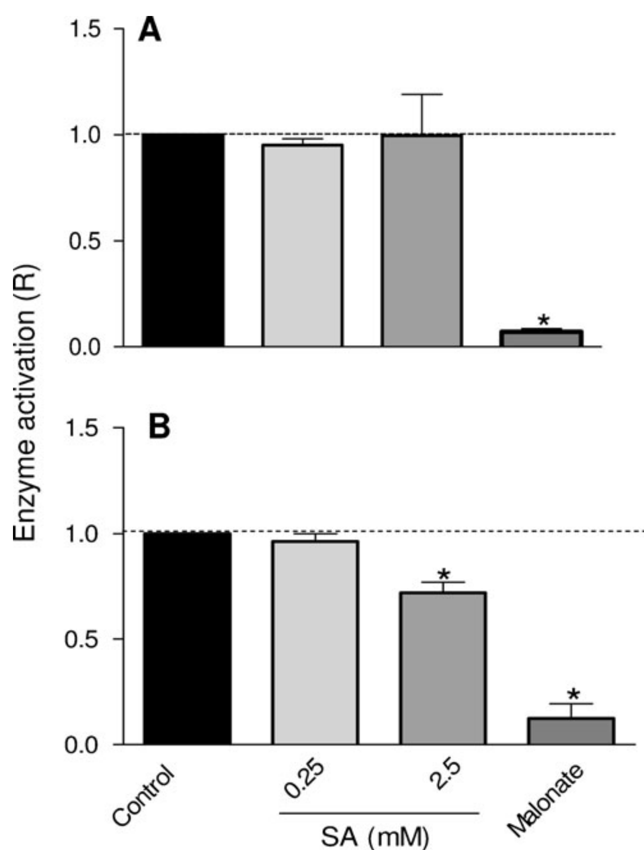

Fig. 5 Effects of SA on MRC activities of mitochondria isolated from cell suspension cultures of *R. fruticosus*. Mitochondria (50 μg of protein) were incubated with SA under the conditions previously described. **a** Succinate ubiquinone reductase activity was measured by the decrease in Abs_{600nm} resulting from the reduction of DCPIP (80 μM); the reaction was initiated with decylubiquinone (80 μM), which served as an electron acceptor. **b** Succinate cytochrome *c* reductase activity was measured by the increase in Abs_{550nm} resulting from the reduction of cytochrome *c* (40 μM). Malonate (10 mM) was used as the control. The results are expressed as R, which represents the relation between the controls (R=1.0) and treated samples (*p< 0.001 vs. control, from 5 independent experiments)

Computational studies on SA-Q-cycle interaction

To propose a potential interaction between SA and the Q cycle, we have employed quantum mechanical calculations. The electron affinities (EA) of SA and ubiquinone were initially assessed. EA could be estimated by determining the energy released after the association of an electron and a neutral molecule. This thermodynamic measurement reflects the stability of the produced anion and indicates the probability of an organic molecule to be reduced. To obtain the EA values, two redox reactions involving SA and ubiquinone were proposed (Fig. 6a); the EA values for ubiquinone and SA were computed as being 2.06 and 0.33 eV, respectively. Because 1 eV corresponds to ~23.05 kcal/mol, we concluded that the energy released during the ubiquinone and SA reductions are 47.48 and 7.60 kcal/mol, respectively. Therefore, ubiquinone reduces

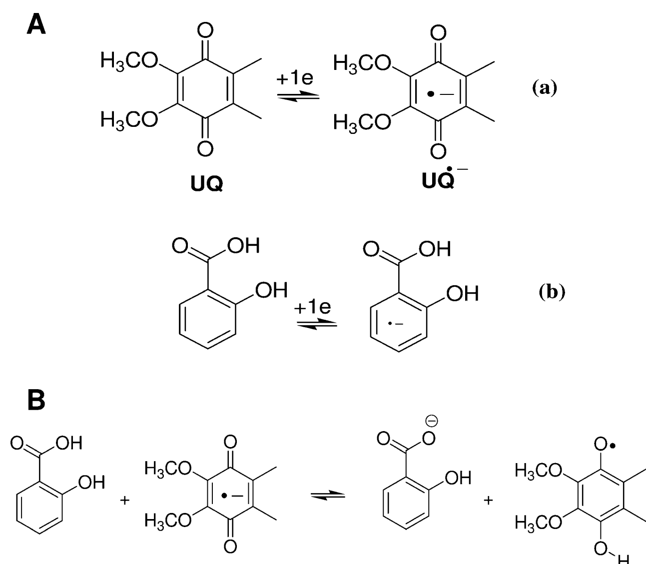

Fig. 6 SA-UQ chemical interactions studies. **a** The proposed redox reactions for ubiquinone (UQ, a) and salicylic acid (SA, b) are displayed. The estimated electronic affinity (EA) values were 2.06 and 0.33 eV, respectively. The energy released during the redox reactions of UQ and SA were 47.48 and 7.60 kcal/mol, respectively, assuming that 1 eV corresponds to 23.05 kcal/mol. **b** The reaction involving the acidic proton transfer from SA to UQ^{-}• is shown. The estimated enthalpy variation for this reaction was −18.37 kcal/mol. The Gibbs energy for this reaction was computed as −20.08 kcal/mol

much easier than SA, suggesting that SA does not interfere with MRC through a direct electron transfer. However, as a weak acid, SA is deprotonated in the physiological pH of the mitochondrial intermembrane space (Shintaku et al. 2007). Therefore, SA is able to accept the protons released from the matrix in energized mitochondria. Due to its hydrophobic nature, the protonated form of SA could cross the inner mitochondrial membrane and interact with its components, for example, donating protons to UQ^{-}•. To verify this possibility, we have calculated the enthalpy and Gibbs energy of the proton transfer from SA to UQ^{-}•. As such, the acidic proton from SA may be easily transferred to UQ^{-}•, indicated by the negative values of enthalpy and Gibbs energies for this reaction, −18.37 and −20.08 kcal/mol, respectively (Fig. 6b). These findings correlate to our above proposal that SA inhibits ROS generation by preventing Q^{-}• accumulation during the Q cycle, as well as by inactivating the radical through its proton transfer.

Discussion

Mitochondria isolated from cell suspension cultures of *R. fruticosus* respiring with NAD^{+}- or FAD-linked substrates are functional, well-coupled and present the alternative enzymes, as well as the PUMP, of typical plant mitochondria (external NAD(P)H dehydrogenases and alternative oxidase). The protocol employed for organelle isolation was satisfactory, leading us to consider mitochondria isolated from cell suspension cultures of *R. fruticosus* as a new model for mitochondrial studies in plants. It was, therefore, employed to assess the mitochondrial sites of ROS generation and the role of SA on mitochondria in plants.

ROS, whose primary generation sites in non-photosynthesizing plant tissues are mitochondria, are involved in the physiological responses, including the resistance of plants to many diseases (Møller 2001; Lam 2008). In the mitochondria isolated from non-photosynthesizing *R. fruticosus* cells, the MRC Complex III inhibitor, AA, stimulated ROS generation while the complex IV inhibitor, KCN, inhibited such generation, either in the absence or presence of AA. It is well established that AA, by binding to cytochrome *b,* increases the steady-state levels of Q^{-}•, which is the chemical species responsible for monovalent reduction of molecular oxygen to form the superoxide radical in the Complex III. By preventing electrons to reach molecular oxygen, KCN inhibits the increased superoxide production that is promoted by AA (Turrens et al. 1985; McLennan and Degli Esposti 2000). The Complex I inhibitor, rotenone, did not stimulate ROS generation. This led us to exclude any involvement of the reverse electron transfer, which has been shown to increase in the succinate-respiring mitochondria exposed to this inhibitor (Tahara et al. 2009). Therefore, our results suggest that ROS generation in our model occurs at the Complex III level.

Salicylic acid (SA) is a signaling molecule and is responsible for many physiological responses in plants (Shirasu et al. 1997; Alvarez 2000; Klessig et al. 2000; Durrant and Dong 2004); its involvement in PCD, particularly in HR, has been proposed (Mur et al. 2008). HR implies in cell death around the site of pathogen infection, restricting the spread of the disease (Mur et al. 2008); ROS production is probably involved in this process (Lam 2008). SA and ROS are thought to act in a cooperative manner to induce physiological responses in plants, with SA either positively or negatively regulating ROS generation (Alvarez 2000; Mur et al. 2008). In addition, SA inhibits mitochondrial respiration, decreases ATP production and induces AOX genes in tobacco cells (Xie and Chen 1999; Norman et al. 2004). Thus, we have demonstrated that SA inhibits state 3 respiration, dissipates $\Delta\Psi$, partially inhibits succinate cytochrome *c* reductase activity and decreases AA-induced ROS generation in *R. fruticosus* mitochondria. These results suggest that SA interacts with MRC Complex III. The concentration range for these effects (0.5–2.5 mM) are commonly observed inside plant cells where SA appears to accumulate rapidly (less than 1 h), even when applied exogenously at low micromolar concentrations (Norman et al. 2004, our unpublished results).

It has been reported that SA prevents the interactions between mitochondrial dehydrogenases and the Q cycle in tobacco cells and that such effect is reversed by mitochondrial washing (Norman et al. 2004); However, a mechanism for these interactions has not been proposed. Our results suggest that SA interacts with MRC via the ubiquinone pool, and a mechanism based on computational energy studies is proposed. We believe that SA inactivates Q^{-}• by an acidic proton transfer and hinders electron flow through MRC, thereby decreasing ROS generation at Complex III. In fact, the evidence that succinate cytochrome *c* reductase activity was not fully inhibited by SA implies that this action does not include any direct interactions of SA with Complex III but, rather, an interference with the Q cycle. Despite the evidence that SA increases ROS generation in many plant systems, this response is thought to occur outside the plant mitochondria (Durner and Klessig 1995; Slaymaker et al. 2002). Our results, however, suggest that the mitochondria are involved and that the Q cycle is the target for SA.

The spatiotemporal pattern of SA accumulation in HR suggests that SA contributes to the controlled timing and extent of cell death through a complex pathway (Alvarez 2000). The survival of tissues surrounding the dead cells found in HR has been associated with the cells' ability to produce both pro-death and anti-death signals (Mur et al.

2008). In contrast to the animal models, the involvement of mitochondria in plant PCD has not been well characterized (Chivasa and Carr 1998; Lam et al. 2001; Mur et al. 2008). Therefore, the action of SA on plant mitochondria, as previously demonstrated and shown here (Xie and Chen 1999; Norman et al. 2004), may help to rationalize the control of plant HR.

Acknowledgement This study was supported by CAPES and CNPq. Wagner R. De Souza received a doctorate scholarship from CAPES and a one-year sandwich CNPq scholarship. Sérgio A. Uyemura and Carlos Curti are fellows from CNPq. Ricardo Vessecchi thanks FAPESP for scholarship. We thank Dr. Luciane C. Alberici for reading and valuable comments on this manuscript; Ana Cristina M. Polizello and Alcides S. Pereira for technical support.

References

Akerman KE, Wikström MK (1976) FEBS Lett 68(2):191–197
Alvarez ME (2000) Plant Mol Biol 44(3):429–442
Barja G (1999) J Bioenerg Biomembr 31(4):347–366
Barrientos A (2002) Methods 26(4):307–316
Becke AD (1993) J Chem Phys 98(7):5648–5652
Bi YM, Kenton P, Mur L, Darby R, Draper J (1995) Plant J 8(2):235–245
Blackstone NW, Green DR (1999) Bioessays 21(1):84–88
Bozhkov PV, Suarez MF, Filonova LH, Daniel G, Zamyatnin AA Jr, Rodriguez-Nieto S, Zhivotovsky B, Smertenko A (2005) Proc Natl Acad Sci USA 102(40):14463–14468
Cape JL, Bowman MK, Kramer DM (2006) Phytochem 67(16):1781–1788
Chivasa S, Carr JP (1998) Plant Cell 10(9):1489–1498
Davis KA, Hatefi Y (1971) Biochemistry 10(13):2509–2516
Dröse S, Brandt U (2008) J Biol Chem 283(31):21649–21654
Durner J, Klessig DF (1995) Proc Natl Acad Sci USA 92(24):11312–11316
Durrant WE, Dong X (2004) Annu Rev Phytopathol 42:185–209
Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Montgomery JA Jr, Vreven T, Kudin KN, Burant JC, Millam JM, Iyengar SS, Tomasi J, Barone V, Mennucci B, Cossi M, Scalmani G, Rega N, Petersson GA, Nakatsuji H, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Klene M, Li X, Knox JE, Hratchian HP, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Ayala PY, Morokuma K, Voth GA, Salvador P, Dannenberg JJ, Zakrzewski VG, Dapprich S, Daniels AD, Strain MC, Farkas O, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Ortiz JV, Cui Q, Baboul AG, Clifford S, Cioslowski J, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Gonzalez C, Pople JA (2004) Gaussian, Inc., Wallingford CT. Gaussian, Inc., 2866
Fukuda H (2004) Nat Rev Mol Cell Biol 5(5):379–391
Hariharan PC, Radom L, Pople JA, Schleyer PVR (1974) J Am Chem Soc 96:599–601
Hehre WJ, Ditchfield R, Pople JA (1972) J Chem Phys 56:2257
Hustache G, Mollard A, Barnoud F (1975) CR Acad Sci 281:1381–1384
Juszczuk IM, Rychter AM (2003) Acta Biochim Pol 50(4):1257–1271
Klessig DF, Malamy J (1994) Plant Mol Biol 26(5):1439–1458
Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P, Trifa Y, Pontier D, Lam E, Silva H (2000) Proc Natl Acad Sci USA 97(16):8849–8855
Kushnareva Y, Murphy AN, Andreyev A (2002) Biochem J 368(Pt 2):545–553
Lam E (2008) CRC Crit Rev Plant Sci 27:413–423
Lam E, Kato N, Lawton M (2001) Nature 411(6839):848–853
Lambert AJ, Brand MD (2004) J Biol Chem 279(38):39414–39420
Lee C, Yang W, Parr RG (1988) Phys Rev B 37:785–789
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193(1):265–275
Mariano AB, Kovalhuk L, Valente C, Maurer-Menestrina J, Pereira-Netto AB, Guerra MP, Carnieri EGS (2004) Braz Arch Biol Technol 47(6):873–879
Maxwell DP, Nickels R, McIntosh L (2002) Plant J 29(3):269–279
McLennan HR, Degli Esposti M (2000) J Bioenerg Biomembr 32 (2):153–162
Mertius S, Tomasi J (1982) Chem Phys 65:239
Møller IM (2001) Annu Rev Plant Physiol Plant Mol Biol 52:561–591
Morel JB, Dangl JL (1997) Cell Death Differ 4(8):671–683
Mur LA, Kenton P, Lloyd AJ, Ougham H, Prats E (2008) J Exp Bot 59(3):501–520
Norman C, Howell KA, Millar AH, Whelan JM, Day DA (2004) Plant Physiol 134(1):492–501
Petrussa E, Braidot E, Nagy G, Vianello A, Macrì F (1992) FEBS Lett 307(3):267–271
Petrussa E, Bertolini A, Krajňáková J, Casolo V, Macrì F, Vianello A (2008) Plant Cell Rep 27(1):137–146
Raskin I, Ehmann A, Melander WR, Meeuse BJ (1987) Science 237 (4822):1601–1602
Rasmusson AG, Soole KL, Elthon TE (2004) Annu Rev Plant Biol 55:23–39
Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Plant Cell 8(10):1809–1819
Shintaku K, Arima Y, Dan Y, Takeda T, Kogushi K, Tsujimoto M, Nagata H, Satoh S, Tsukimori K, Nakano H, Hori S, Ohtani H, Sawada Y (2007) Drug Metab Dispos 35(5):772–778
Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C (1997) Plant Cell 9(2):261–270
Slaymaker DH, Navarre DA, Clark D, del Pozo O, Martin GB, Klessig DF (2002) Proc Natl Acad Sci USA 99(18):11640–11645
Tahara EB, Navarete FD, Kowaltowski AJ (2009) Free Radic Biol Med 46(9):1283–1297
Trumpower BL (1990) J Biol Chem 265(20):11409–11412
Turrens JF (2003) J Physiol 552(Pt 2):335–344
Turrens JF, Boveris A (1980) Biochem J 191(2):421–427
Turrens JF, Freeman BA, Levitt JG, Crapo JD (1982) Arch Biochem Biophys 217(2):401–410
Turrens JF, Alexandre A, Lehninger AL (1985) Arch Biochem Biophys 237(2):408–414
Vercesi AE, Borecký J, Maia IG, Arruda P, Cuccovia IM, Chaimovich H (2006) Annu Rev Plant Biol 57:383–404
Xie Z, Chen Z (1999) Plant Physiol 120(1):217–226