Scientific Correspondence

Control of Ascorbate Synthesis by Respiration and Its Implications for Stress Responses[1]

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We show for the first time that respiration can control ascorbate (AA) synthesis in plants. Evidence for this control is provided by (a) the localization of L-galactono-1,4-lactone dehydrogenase (GalLDH), the terminal enzyme in AA biosynthesis, with mitochondrial complex I, and its regulation by electron transport through this complex, (b) the absolute requirement of the enzyme for oxidized cytochrome c (cyt $c_{(ox)}$) as substrate, and (c) the coordinated response of respiration and AA synthesis to stress induced by hormone treatment.

AA is a high abundance metabolite in plants with key roles in plant development and stress tolerance. Therefore, triggers that modulate respiration may also impact on AA production and hence stress responses. Unlike terminal enzymes of AA synthesis found in animals and fungi, plant GalLDH does not produce H_2O_2 as a by-product (Smirnoff, 2001). This might have evolved to avoid excessive oxidation of the mitochondrial electron transport chain (ETC) and damage to tricarboxylic acid cycle enzymes, because mitochondria are very susceptible to oxidative inhibition of function (Verniquet et al., 1991; Nulton-Persson and Szweda, 2001; Sweetlove et al., 2002). Moreover, recent evidence also suggests that the redox state of the plant mitochondrial ETC is critical in setting the whole cell redox stat (Dutilleul et al., 2003).

It has been shown by ourselves and other authors that GalLDH is an integral protein of the inner mitochondrial membrane (Siendones et al., 1999; Bartoli et al., 2000) and that cyt c is the electron acceptor for the GalLDH reaction. We have demonstrated previously (Bartoli et al., 2000) that the availability of cyt $c_{(ox)}$ modulates AA synthesis in isolated, intact potato (*Solanum tuberosum*) mitochondria, as might be expected in a simple relationship based on enzyme kinetics. In these experiments, the rate of AA synthesis was completely inhibited by KCN, which blocks cyt c oxidation, but not by antimycin A, which prevents cyt c reduction, thus altering the availability of the GalLDH substrate, cyt $c_{(ox)}$ (Bartoli et al., 2000). Rotenone had no effect on AA synthesis under these conditions (Bartoli et al., 2000). It is well accepted that in both plant and animal mitochondria, cyt c redox poise responds to respiratory inhibitors before its site of electron acceptance (such as rotenone and antimycin A) by becoming more oxidized, and by inhibitors following it (such as KCN and CO) by becoming more reduced (see Storey, 1980; Halestrap, 1982, and refs. therein).

In the present experiments, AA synthesis in Arabidopsis mitochondria is also dependent on the availability of cyt $c_{(ox)}$ for GalLDH activity when tricarboxylic acid cycle substrates pyruvate and malate are used to drive electron transport (Table I). However, in this case, we also demonstrate that rotenone, a specific inhibitor of complex I, inhibits AA synthesis rate to the same degree as KCN (Table I). As rotenone oxidizes the cyt c pool, this inhibition of AA synthesis cannot be explained by the simple substrate kinetics invoked to interpret the effects of KCN and antimycin A. When complex I is not engaged, there is no effect of added rotenone on the rate of AA synthesis (Table II). This observation was made when electrons entered the chain via succinate dehydrogenase (by the addition of succinate). This agrees with the results of the previous study, where there was no effect of added rotenone when external NADH dehydrogenases were engaged by addition of exogenous NADH; Bartoli et al., 2000). Similarly, when electrons are introduced only via GL to cytochrome c (by adding no substrate other than GL), there is no effect of added rotenone on AA synthesis rate (Table II), but the rate of AA synthesis is lower than when complex I is operating (Table I). This strongly suggests that electron flow through complex I affects AA synthesis rate and that this electron flow is required for optimal rates of AA synthesis. This clearly implies a novel and intriguing relationship between complex

[1] This work was supported by grant-aided support to Rothamsted Research from the Biotechnology and Biological Science Research Council of the UK and by Fundación Antorchas (Argentina).

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www.plantphysiol.org/cgi/doi/10.1104/pp.103.028399.

Table I. *The differential effect of respiratory inhibitors on AA synthesis and malate/pyruvate-dependent oxygen consumption by isolated Arabidopsis mitochondria*

Mitochondria were isolated from leaves of Arabidopsis plants according to Purvis (1997), and oxygen uptake was measured with a Clark oxygen electrode. The reaction was conducted with 100 μg of mitochondrial protein in 1.5 mL of respiration buffer containing 10 mM MOPS, pH 7.2, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 0.5 mg mL^{-1} BSA, 250 mM Suc. Concentrations of substrates were as indicated; solvent controls were not significantly different from untreated controls. State 3 respiration rates were established with ADP before addition of the inhibitors. AA production was determined in respiration buffer containing 200 μg of mitochondrial protein and 2 mM L-galactono-lactone (GL). Reaction mixtures were placed in a water bath at 25°C for 30 min, and the reaction was stopped by the addition of 1 volume of 1 M $HClO_4$. AA content was determined as described by Bartoli et al. (2000). Differences from control values were significant (according to Student's *t* test) at: (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$; (d) not significant. Mitochondrial intactness was determined before and after incubation, by measuring cytochrome c oxidoreductase activity in the presence or absence of 0.01% (w/v) Triton X-100, as described by Schwitzguebel and Siegenthaler (1984) and calculated according to the formula [100− (activity associated with intact organelle/activity associated with permeabilized × 100)] (Burgess et al., 1985). Cross-contamination of mitochondria by chloroplasts was 1.2% ± 0.1%, as judged by chlorophyll (Arnon, 1949). Cross-contamination by peroxisomes was 9.5% ± 1.21%, using catalase as a marker (Rao et al., 1996).

	Oxygen Consumption nmol O_2 min^{-1} mg^{-1} protein	AA Production nmol mg^{-1} protein	Intactness	
			0 min	30 min
			%	
2 mM GL	27.25 ± 4.59	151.38 ± 25.65	85 ± 5	82 ± 3
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL	132.01 ± 10.25	281.67 ± 49.30[a]	85 ± 5	83 ± 4
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL + 1 mM KCN	0	39.25 ± 3.02[b]	85 ± 5	79 ± 5
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL + 1.5 μM anti A	24.82 ± 2.19	186.97 ± 36.60[d]	85 ± 5	76 ± 4
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL + 50 μM rotenone	1.16 ± 0.15	21.21 ± 1.13[c]	85 ± 5	72 ± 6

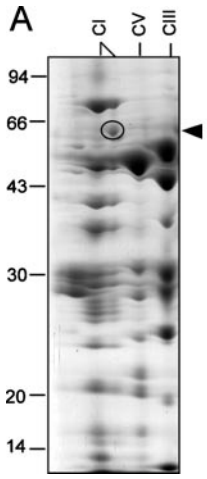
I and GalLDH that has not been documented previously.

Recently, proteomic techniques have revealed a potential structural basis for the functional relationship described above. A combination of Blue Native PAGE coupled to a second dimension of denaturing SDS-PAGE and mass spectrometry has been used to identify many of the components of complexes I, III, and V (Heazlewood et al., 2003a, 2003b). Critically, there are two types of complex I in Arabidopsis mitochondria (Fig. 1A): a minor high mobility form and a major lower mobility form. The higher mobility form contains an additional subunit of approximately 62 kD (Fig. 1A) that is absent from the major lower mobility form. Sequencing of peptides derived from this protein by collision-induced dissociation and mass spectrometry revealed seven separate peptides that matched to the sequence of the Arabidopsis GalLDH (GenBank accession no. BAA95212; Fig. 1B). These peptides cover 84 amino acids (or 14%) of the GalLDH amino acid sequence. A combined MOWSE score of 238 reveals this is a highly significant match ($P < 0.05$ when MOWSE > 47). Although comprehensive proteomic analyses have been conducted, GalLDH was not identified in any other complex or in sub-compartment fractions of mitochondria (Heazlewood et al., 2003a, 2003b; Millar et al., 2001). In some reports, small amounts of GalLDH activity have been detected in soluble fractions extracted from plant tissue. Given that membranes were not exhaustively removed in these studies by high speed centrifugation, this protein cannot be classified as soluble. Hence GalLDH is physically associated with complex I in Arabidopsis, even under the fairly stringent high-salt and nonionic detergent conditions of Blue Native-PAGE. The localization of this enzyme on the minor high mobility form of the complex may further indicate that there is a sub-population of

Table II. *Effect of rotenone on production of ascorbate by isolated Arabidopsis mitochondria using non-complex I respiratory substrates*

Mitochondrial isolation, oxygen consumption, and ascorbate synthesis rate were determined as indicated in Table I. Differences from control values were significant (according to Student's *t* test) at: (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$; (d) not significant.

	Oxygen Consumption nmol O_2 min^{-1} mg^{-1} protein	AA Production nmol mg^{-1} protein	Intactness	
			0 min	30 min
			%	
2 mM GL	27.25 ± 4.59	151.38 ± 25.65	85 ± 5	82 ± 3
2 mM GL + 2 mM ADP + 50 μM rotenone	0	160.90 ± 18.79[d]	85 ± 6	83 ± 7
10 mM succinate + 2 mM ADP + 2 mM GL	192.98 ± 8.03	160.31 ± 25.17[d]	85 ± 7	84 ± 9
10 mM succinate + 2 mM ADP + 2 mM GL + 50 μM rotenone	182.20 ± 21.45	152.37 ± 28.24[d]	85 ± 5	73 ± 6
1 mM NADH + 2 mM ADP + 2 mM GL	242.98 ± 21.30	149.75 ± 15.79[d]	85 ± 5	81 ± 6



B

m/z	Mr	MOWSE	Sequence match to GLDH (BAA95212)
[566.27]$^{+1}$	565.26	9	DLYR
[480.24]$^{+2}$	958.46	32	VLEVDKEK
[640.30]$^{+2}$	1278.59	55	DLEYIEELKK
[659.35]$^{+2}$	1316.67	28	YTTDEAVQHVR
[671.32]$^{+2}$	1340.63	36	DITDEFFHYR
[677.81]$^{+2}$	1353.60	27	EKDPELFHLAR
[759.39]$^{+2}$	1516.76	21	LLALDPLNDVHVAK
[908.38]$^{+2}$	1814.74	28	NFNQPENLADLEALVK

84 AA residues, 14% coverage, MOWSE total = 236

Figure 1. Blue Native PAGE of mitochondrial proteins from Arabidopsis. A, Mitochondria were isolated from an Arabidopsis cell culture according to Millar et al. (2001), and 1 mg of membrane protein was subjected to Blue Native PAGE according to Jänsch et al. (1996) as modified by Heazlewood et al. (2003a). After this first dimension separation, lanes were cut from gels, equilibrated in standard SDS-PAGE sample buffer for 30 min, laid horizontally on 12% (w/v) acrylamide SDS-PAGE separating gels, and sealed in with 0.5% (w/v) agarose; and electrophoresis was continued for 5 h under standard conditions. Proteins were visualized by colloidal Coomassie staining. Complexes I, V, and III are shown. B, Mass spectroscopic analysis. The protein spot identified by a circle in A was cut from the gel, digested with trypsin according to Sweetlove et al. (2001) and injected into the electrospray source in 50% (v/v) methanol/0.1% (v/v) formic acid. Selected doubly charged peptides, identified in mass spectrometry-time-of-flight (mass spectrometry-TOF) mode, were fragmented by N_2 collision analyzed by Q-TOF tandem mass spectrometry (MS/MS) on a Q-STAR Pulsar (Q-TOF MS; Applied Biosystems, Sydney) using an IonSpray source. Mass spectra and collision MS/MS data were analyzed with Analyst QS software (Applied Biosystems) and searched against the equivalent theoretical masses derived from the National Center for Biotechnology Information protein database using MASCOT (http://www.matrixscience.com). Mass spectra, collision MS/MS data, and sequence matches to galactono-1,4-lactone dehydrogenase (GLDH) are shown.

complex I whose function may include regulating AA synthesis by monitoring the rate of NADH-driven electron flow through complex I.

Although the molecular mechanism linking the rotenone inhibition of complex I to GalLDH catalysis (Table I) and to GalLDH as a complex I component (Fig. 1) remains to be elucidated, possible interactions can be postulated. Rotenone is believed to act by binding to the 20-kD PSST 4Fe-4S binding subunit of complex I (NuoB in the bacterial enzyme) prevent-

Table III. *Effect of GA treatment on ascorbate synthesis and malate/pyruvate-dependent oxygen consumption by isolated Arabidopsis mitochondria*

Four-week-old Arabidopsis plants were sprayed with 100 μM GA_3; each plant received approximately 30 nmol. Mitochondria were isolated 3 d after treatment, and oxygen consumption and ascorbate synthesis rate were determined as indicated in Table I. Differences from control values were significant (according to Student's *t* test) at: (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$.

	Control Plants				GA-Treated Plants			
	Oxygen Consumption nmol O_2 min^{-1} mg^{-1}	AA Production nmol mg^{-1} protein	Intactness		Oxygen Consumption nmol O_2 min^{-1} mg^{-1}	AA Production nmol mg^{-1} protein	Intactness	
			0 min	30 min			0 min	30 min
	protein		%		*protein*		%	
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL	139.25 ± 18.56	275.32 ± 25.44	86 ± 9	81 ± 10	79.31 ± 9.85	886.61 ± 79.30[c]	87 ± 7	80 ± 8
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL + 50 μM rotenone	2.25 ± 0.31	8.88 ± 2.21	86 ± 9	76 ± 8	0	19.42 ± 2.66[b]	87 ± 7	71 ± 6
2 mM ADP + 10 mM malate + 10 mM pyruvate + 2 mM GL + 2 mM KCN	0	24.35 ± 2.98	86 ± 9	79 ± 5	0	38.99 ± 4.55[b]	87 ± 7	81 ± 9
2 mM ADP + 10 mM succinate + 2 mM GL	189.62 ± 21.27	269.85 ± 24.32	86 ± 9	85 ± 8	111.55 ± 7.88	693.44 ± 77.25[c]	87 ± 7	84 ± 9
2 mM ADP + 10 mM succinate + 2 mM GL + 50 μM rotenone	178.69 ± 33.51	251.33 ± 31.26	86 ± 9	72 ± 7	105.33 ± 11.28	658.99 ± 59.63[c]	87 ± 7	79 ± 6

ing further electron transport to the membrane arm of complex I, leading ultimately to ubiquinone reduction (Nicholls and Ferguson, 2001). One explanation might be that there is a requirement for a particular redox status of a Fe-S center or the flavin center upstream or downstream of NuoB. Alternatively, conformational changes induced in complex I by rotenone binding alter the redox poise in such as way as to prevent GalLDH catalysis. The discovery of the phosphorylation of the 18-kD subunit of complex I (AQDQ subunit) in mammals and its functional effects on complex I (Papa et al., 1996) suggest that posttranslational modification and perhaps even resultant conformation changes could function in complex I. Similar mechanisms may also explain the relationships described here (Tables I and II). The recent evidence that complex I actually exists in supercomplexes with complex III in plant mitochondria suggests another layer of regulation of the ETC making the "front end" of the respiratory chain from NADH to reduced cyt c a single functional unit in plant mitochondria (Eubel et al., 2003), as it is in mammalian mitochondria (Schägger and Pfeiffer, 2000). In this context, GalLDH could be viewed as being structurally and functionally "attached" to the native cyt c-reducing supercomplex of the respiratory chain in plants. Together with this evidence, our observations suggest that much remains to be uncovered about the subtleties of complex I function. Even in the absence of a defined mechanism, the discovery of a physical and functional link between complex I and AA synthesis is important.

We have therefore posed the question of whether AA synthesis is regulated in plants by mitochondrial function. Given the results of substrate feeding experiments (Loewus, 1963; Pallanca and Smirnoff, 2000), it has been assumed to date that the supply of GL to the mitochondrion is the major limitation on AA production and accumulation in plants. GA_3 is known to decrease respiration in plants (Franco and Han, 1997; Nagel and Lambers, 2002), the as yet untested assumption being that this allows conservation of carbon substrates during periods of rapid growth. We isolated mitochondria from plants sprayed with GA_3 and examined the relationship between electron transport rate and AA synthesis. As predicted, the rate of oxygen consumption was decreased in mitochondria from GA-treated plants, but a clear stimulation of AA synthesis was observed (Table III). However, the regulation of AA synthesis via electron transport substrates and inhibitors was identical in mitochondria from control and GA-treated plants. The stimulation of AA synthesis must result from enhanced GalLDH activity, which could be explained either by increased GalLDH protein or increased catalysis. Regardless of the mechanism, these data establish a clear relationship between the rate of respiration and AA synthesis. This opens up a new field of investigation because there is a clear implication that stress tolerance is linked to mitochondrial function (Dutilleul et al., 2003). Because mitochondria are known to be a site of initiation of cell death and a component of complex I has been implicated in this process (Fearnley et al., 2001), it is intriguing to speculate that modulation of the production of the major redox buffer of plant cells could be part of the intricate control network that determines the fate of the cell.

ACKNOWLEDGMENT

V. Mittova thanks the European Molecular Biology Organization for a short-term fellowship.

Received June 10, 2003; returned for revision July 1, 2003; accepted July 1, 2003.

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