Functional and composition differences between mitochondrial complex II in Arabidopsis and rice are correlated with the complex genetic history of the enzyme.

Shaobai Huang, Nicolas L. Taylor, Reena Narsai, Holger Eubel, James Whelan, A. Harvey Millar

ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, M316, Crawley 6009, WA, Australia

Corresponding author:

A.H. Millar, email: harvey.millar@uwa.edu.au, fax: +61 8 6488 4401, ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, M316, Crawley 6009, WA, Australia

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Abstract

Complex II plays a central role in mitochondrial metabolism as a component of both the electron transport chain and the tricarboxylic acid (TCA) cycle. However, the composition and function of the plant enzyme has been elusive and differs from the well-characterised enzymes in mammals and bacteria. Herewith, we demonstrate that mitochondrial Complex II from Arabidopsis and rice differ significantly in several aspects: i) Stability - Rice complex II in contrast to Arabidopsis is not stable when resolved by native electrophoresis and activity staining. ii) Composition - Arabidopsis complex II contains 8 subunits, only 7 of which have homologs in the rice genome. SDH 1 and 2 subunits display high levels of amino acid identity between two species, while the remainder of the subunits are not well conserved at a sequence level, indicating significant divergence. iii) Gene expression - the pairs of orthologous *SDH1* and *SDH2* subunits were universally expressed in both Arabidopsis and rice. The very divergent genes for *SDH3* and *SDH4* were co-expressed in both species, consistent with their functional co-ordination to form the membrane anchor. The plant-specific *SDH5*, 6 and 7 subunits with unknown functions appeared to be differentially expressed in both species. iv) Biochemical regulation - succinate-dependent O₂ consumption and SDH activity of isolated Arabidopsis mitochondria were substantially stimulated by ATP, but a much more minor effect of ATP was observed for the rice enzyme. The ATP activation of succinate-dependent reduction of DCPIP in frozen-thawed and digitonin-solubilised mitochondrial samples, and with or without the uncoupler CCCP, indicate that the differential ATP effect on SDH is not via the protonmotive force but likely due to an allosteric effect on the plant SDH enzyme itself, in contrast to the enzyme in other organisms.

Key words: Rice, Arabidopsis, Mitochondria, Complex II, Succinate dehydrogenase,

Introduction

Succinate:ubiquinone oxidoreductase (succinate dehydrogenase [SDH]; EC 1.3.5.1), commonly known as Complex II in mitochondria, has a central role in mitochondrial metabolism as a component of both the electron transport chain and the tricarboxylic acid (TCA) cycle. Complex II catalyses the oxidation of succinate to fumarate and the reduction of ubiquinone (UQ) to ubiquinol (UQH₂). Classically, SDH contains four subunits: a flavoprotein (SDH1) that houses the FAD cofactor, an iron-sulfur protein (SDH2) containing three Fe-S clusters, and two small integral membrane proteins (SDH3 and SDH4) that bind heme to form a *b*-type cytochrome and also fold together to form the UQ binding site within the membrane (Lemire and Oyedotun, 2002). The SDH structure was first resolved by X-ray crystallography from *E. coli* (Yankovskaya et al., 2003). In mammals, the Complex II crystal structure was also confirmed to consist of two hydrophilic proteins, (flavoprotein and iron-sulfur protein) and the two transmembrane proteins housing the *b*-type cytochrome (CybL and CybS) (Sun et al., 2005). Comparison of SDH/Complex II structure from *E. coli* and mammalian mitochondria have shown substantial differences in the transmembrane region and in environments surrounding the prosthetic groups causing significant shifts in the midpoint redox potential of the enzymes (Sun et al., 2005).

Compared with mammals, higher plants have a divergent Complex II. The enzymes have similar sequences for SDH1 and SDH2, but great divergence in sequences for the membrane anchor proteins SDH3 and SDH4 (Burger et al., 1996). In addition to these four classical SDH subunits, the model dicot plant *Arabidopsis* had four other subunits, presumably plant-specific, which have been resolved by blue native (BN)/SDS-PAGE and identified by mass spectrometry (Eubel et al., 2003; Millar et al., 2004). The four additional Complex II subunits, termed SDH5, 6, 7 and 8, may represent a secondary or peripheral activity of this complex, but none of these extra four proteins have homology to known function proteins in sequence databases (Millar et al., 2004). In other dicot plants, such as potato and bean, Complex II can be separated by BN/SDS-PAGE revealing a very similar pattern to that observed in *Arabidopsis* (Eubel et al., 2003). However, in monocot plants such as barley and rice, Complex II is not observed in BN/SDS-PAGE separation of mitochondrial proteins (Eubel et al., 2003; Heazlewood et al., 2003). Therefore, the potential differences in mitochondrial Complex II structure and function between monocots and dicots required further investigation.

The biochemical characteristics of mitochondrial SDH have been investigated for more than three decades. In mammals, deactivation of SDH enzyme activity after isolation

occurs due to the presence of tightly bound oxaloacetate (Kearney et al., 1972; Ackrell et al., 1974). However, the isolated SDH can be activated by addition of substrates (Kearney, 1957) as well as by a range of anions and by acidic pH (Kearney et al., 1972), by reducing treatments (Ackrell et al., 1975) and by its product, QH₂ (Gutman et al., 1971a, 1971b). Additionally, mammalian SDH is deactivated by ADP and activated by ATP in intact mitochondria, but the ATP activation effect was absent in purified SDH (Gutman et al., 1971a). In higher plants, SDH could be activated by substrates, the product QH₂, anions and acid pH, in a similar pattern to that observed in mammals (Oestreicher et al., 1973; Singer et al., 1973). However, the ATP activation of SDH in intact plant mitochondria is not lost during freezing and sonication of mitochondria (Oestreicher et al., 1973; Singer et al., 1973).

The activation of SDH by adenine nucleotides has been proposed to be mediated indirectly by the proton motive force (Affourtit et al., 2001). This claim was supported by evidence that: (1) by simultaneously measuring the oxygen uptake rate and the UQ redox status, the activation of SDH in isolated potato mitochondria by ATP with uncoupler CCCP was slower than that without CCCP (Affourtit et al., 2001); (2) in monocot *Arum* mitochondria, SDH was not stimulated by ADP or ATP when the membrane potential ($\Delta\psi$) was minimised, but rather it appeared to be deactivated by the adenine nucleotides (Leach et al., 1996); and (3) activation of SDH by both ATP and ADP in the intermembrane space can occur by the indirect inhibition the K⁺ channel and thereby increasing $\Delta\psi$, because ATP activation was insensitive to inhibitors of ATP transport across the inner membrane (Affourtit et al., 2001). However, Affourtit et al (2001) stopped short of concluding that the entire ATP effect on SDH was indirect in plants. Hence, while ATP activation of the mammalian enzyme may be explained by an indirect effect outlined by Affourtit et al (2001), direct ATP stimulation of plant SDH must also be a possible explanation for the results observed at least from dicot species (Oestreicher et al., 1973; Singer et al., 1973). Consistent with a direct route of activation, we have recently shown that SDH1 is an ATP binding protein in the dicot model species, *Arabidopsis* (Ito et al., 2007).

Here we have performed a systematic analysis of protein sequences of mitochondrial Complex II subunits in *Arabidopsis* and rice which has raised questions about differences in Complex II between these model dicot and monocot plant species. Evidence for differential gene expression patterns, protein complex structure and direct adenine nucleotide activation of complex II in the two species is presented.

Materials and Methods

Growth of rice seedlings and mitochondrial preparation

Batches of 200 g rice (*Oryza sativa* L. cv Amaro) seed were washed in 1% (w/v) bleach for 10 min, rinsed in distilled water, and grown in the dark in vermiculite trays (30 x 40 cm) at a constant 30°C, watered daily, and the shoot tissues harvested at 10 d for mitochondrial isolations. Rice mitochondrial isolation were by differential centrifugation followed by Percoll gradient centrifugation (Huang et al., 2009a). After 0-4.4% (w/v) PVP plus 28% Percoll gradient centrifugation, organelles were washed three times with washing medium (0.3M sucrose, 10 mM TES, pH7.5) and centrifuged at 24,000 g for 10 min at 4°C. The amount of mitochondrial protein was determined according to the Bradford method (Bradford, 1976).

Arabidopsis culture cell and mitochondrial preparation

A heterotrophic Arabidopsis cell culture was maintained under the same conditions as described in Millar et al. (2001). Arabidopsis cell suspension was cultured in growth medium (1x Murashige and Skoog medium without vitamins, 87.7 mM sucrose, 26.9 µM naphthaleneacetic acid, 0.23 µM kinetin, pH 5.8) at 22°C under a 16/8-h day/night regime and light intensity of 90 µmol m⁻² s⁻¹ with orbital shaking at 120 rpm. Cultures were maintained in 250 ml Erlenmeyer flasks by the inoculation of 20 ml of 7-10 day old cells into 100 ml of fresh growth medium. Dark-grown cells used for mitochondrial isolation were subcultured from 7day old light-grown cultures and grown under the same conditions as described for light cell cultures. Isolation of mitochondria from cell culture was carried out according to methods described previously (Millar et al., 2001; Huang et al., 2009a). The amount of mitochondrial protein was determined according to the Bradford method (Bradford, 1976).

Protein identifications using LC-MALDI TOF-TOF

Protein samples were analyzed using complex mixture LC-MALDI TOF/TOF analysis. Rice mitochondrial protein extract was digested overnight at 37°C with trypsin and insoluble components were removed by centrifugation at 20000 x g for 5 min. Samples were spotted onto Anchorchips (800-384, Bruker Dlatronics) using a Proteineer FC (Bruker Daltronics). Initially peptides were trapped on a RP-18 capillary, 100µm x 2cm, 5µm (Nanoseparations)

column and then separated on a Pepman 100 (75µm x 15cm, 3µm, Dionex) at 300 nl/min from 2%B-45%B (A: 0.05% TFA in H₂O; B: 0.05%TFA in 90% acetonitrile/10% H₂O). Each spot on the Anchorchip containing sample was obtained from 10 s of RP-18 flow, yielding 768 spots from the gradient. Each was co-eluted with a solution of α-Cyano-4-hydroxycinnamic acid matrix. Mass spectra were recorded on an Ultraflex III TOF/TOF instrument (Bruker Daltonics) equipped with LIFT capability. A pulsed Nd : YAG laser at a wavelength of 355 nm was operated at a frequency of 100 Hz. After spotting the dried spots were washed twice with wash solution (10 mM NH₄H₂PO₄, 0.1% TFA) and the MALDI target was introduced into the mass spectrometer ion source. External calibration was performed with commercial peptide mixture (Peptide calibration standard 1, Bruker Daltonics). Data was acquired with the Flex Control software (v 3.0, Bruker Daltronic). The mass spectra were obtained from the recorded raw data using Flex Analysis software (3.0, Bruker Daltronic) by applying the external calibration protocol. Output files from MALDI-TOF/TOF were combined and data was exported as mgf file. This file was then searched using the Mascot search engine version 2.1.04 (Matrix Science) against the TIGR rice proteome (Osa5.all.pep: TIGR) utilizing error tolerances of ± 100 ppm for MS and ± 0.5 Da for MS/MS, 'Max Missed Cleavages' set to 1, the Oxidation (M), Carboxymethyl (C), variable modifications and the instrument set to MALDI-TOF/TOF and Peptide charge set at 1+. Results were filtered using 'standard scoring', 'Max. number of hits' set to 20, 'Significance threshold' at p< 0.05 and 'Ions score cut-off' at 20.

Succinate-dependent oxygen consumption using O₂ electrode

Oxygen consumption by purified rice and Arabidopsis mitochondria was measured by a computer-controlled Clark-type O₂ electrode unit (Hansatech-Instruments, Pentney, UK). Calibration of the electrode was carried out by addition of sodium dithionite to remove all oxygen in the electrode chamber. The air-saturated oxygen concentration was assumed to be 240 µM at 25°C, which was applied for the following measurements. All reactions were carried out at 25°C using 1 ml of mitochondrial reaction medium (0.3 M sucrose, 10 mM TES, 10 mM NaCl, 4 mM MgSO₄, 0.1 % (w/v) BSA, pH 7.2) and approximately 100 µg of mitochondrial protein. To investigate the succinate-dependent O₂ consumption, 5 mM succinate was added to the reaction solution. To investigate the activation of succinate-dependent O₂ consumption by ATP, ATP was added to the reaction solution at a final concentration of 0.5 mM.

SDH activity assay using spectrophotometry

The SDH activity was determined from a modified version of the method from Singer et al. (1973). The mitochondria fraction (~30 µg protein) was assayed for activity spectrophotometrically at 600 nm at 30°C, in 1 ml of a reaction medium (50 mM potassium phosphate pH 7.4, 10 mM sodium succinate, 0.1 mM EDTA, 0.1% (w/v) BSA, 10 mM potassium cyanide, 0.12 mM dichlorophenolindophenol (DCPIP), 1.6 mM phenazine methosulfate). For ATP activation experiments, the ATP was added into the 1 ml reaction solution to a final concentration of 0.5 mM. To test whether ATP activation was dependent on membrane potential or not, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to 1 ml reaction solutions to a final concentration of 4 µM. An extinction coefficient of 21 mM⁻¹ cm⁻¹ at 600 nm for DCPIP was used for calculations.

Blue-native and clear-native gel and Complex II activity staining

The method for blue-native (BN) gel electrophoresis and Complex II activity staining were adapted from (Eubel et al., 2005) and Jung et al. (2000) with some modifications. Mitochondria aliquots (1 mg protein) were centrifuged for 10 min at 14,300 g, and pellets were resuspended in the BN re-suspension buffer (pH 7.4, 30 mM HEPES, 150 mM potassium acetate, 10% glycerol) with different detergents as shown in Figure and incubated on ice for 20 min. After centrifugation at 18300 g for 20 min, the supernatants were transferred into a new Eppendorf tube and 1 µl of 5% 'Serva Blue G' solution (750 mM caproic amino acid, 5% Coomassie blue 250 G) was added. After mixing, the solutions were transferred onto the gel. The first dimension BN gel electrophoresis and second SDS-PAGE electrophoresis were carried out according to the methods of Eubel et al. (2005). For complex II activity staining, the first dimension BN-native gel were soaked in staining medium (50 mM potassium phosphate buffer, pH 7.5, 84 mM succinate Na, 0.2 mM phenazine methosulfate, 2 mg/ml nitro tetrazolium blue, 4.5 mM EDTA, 10 mM potassium cyanide) as described in Eubel et al. (2005) and (Jung et al., 2000). After 2-3 h of staining, the gels were scanned. The method for clear-native (CN) gel electrophoresis was similar to the above BN method except without any addition of Coomassie blue 250 G during sample preparation or to the gel running buffer. The methods for the Complex II activity staining and second dimension electrophoresis were the same as those used for analysis of the BN gels.

Comparison of public Arabidopsis and rice microarray data

In order to compare the transcript abundance changes of genes encoding Complex II subunits across different tissues and conditions, Arabidopsis and rice array data were

retrieved from the Gene expression Omnibus within the National Center of Biotechnology Information database (NCBI). In Fig. 1, the array data is represented as numbered columns. For Arabidopsis, these datasets were: Nakabayashi et al., 2005 (1-4); GSE12676 (5-8); GSE5632 (9-14); GSE3065 (15-17); GSE680 (18-28); GSE5751 (19-30); E-ATMX-31 (31-33). For rice, the datasets were E-MEXP-1766 (1-5); GSE6908 (6-7); GSE6908 (6-7); GSE6893 (8-22); GSE6901 (23); GSE7965 (24-32). As was also reported in Huang et al. (2009b), all data were MAS5.0 normalized and normalized against average ubiquitin expression. The normalized array data were then compiled together and for each probe set, the maximum expression was set to 1.0, with all other data relative to this. This facilitates cross-comparison of arrays from all of the different studies at once. Partek Genomic suit software (version 6.4) was used to carry out hierarchical clustering based on Euclidian distance.

Results

Mitochondrial Complex II subunits in Arabidopsis and rice

The mitochondrial complex II in Arabidopsis has eight subunits (Eubel et al., 2003; Millar et al., 2004) and we have recently identified six SDH subunits in an in-depth analysis of the rice mitochondrial proteome (Huang et al., 2009b). Here we combine a sequence analysis in the genomes of the two species (Table 1) with a further mass spectrometry (MS) analysis of rice mitochondria using a LC MALDI-TOF/TOF approach (Table 2) to compare Complex II composition. There are two copies of the *SDH1* gene encoding the SDH flavoprotein in the Arabidopsis genome, but only a single copy in the rice genome (Table 1). The SDH1 subunits identified by MS (At5g66760 and Os07g04240) are nearly 90% identical at the amino acid level, demonstrating a high degree of conservation of this flavoprotein between rice and Arabidopsis.

There are three copies of *SDH2* in the Arabidopsis genome and two proteins (At3g27380 SDH2-1; At5g40650 SDH2-2) have been previously identified in mitochondria by mass spectrometry (Table 1). The SDH2-3 isoform (At5g65165) that has not been detected by mass spectrometry only has 67% identity to SDH2-1 and -2, and has a divergent N-terminal sequence. In rice, we identified Os08g02640 to encode SDH2 and it has 70% identity to Arabidopsis SDH2-1 and -2 (Table 1). Os08g02640 encodes both a single functional mitochondrial SDH2 and also a chimeric SDH2-RPS14 protein processed by alternative splicing (Kubo et al., 1999). A similar chimeric for SDH2 has also been observed in maize (Figueroa et al., 1999; Figueroa et al., 2000). Our new TOF-TOF MS/MS data provides evidence that both forms co-exist in rice mitochondria based on identification of gene-specific peptides unique to each isoform (Table 2, Supplemental Fig. 1). The rice genome also has another putative *SDH2* gene (Os09g20440, Table 1), with 56% identity to Os08g02640, but with an additional 380 aa N-terminal sequence that lacks similarity to other SDH2 sequences. Os09g20440 is more similar to the third isoform in Arabidopsis, At5g65165 (SDH2-3), than to the other two Arabidopsis isoforms. It is possible that this represents another chimeric involving SDH2 and an unknown function protein.

There is great divergence in the sequences for the hydrophobic membrane anchor proteins SDH3 and SDH4. The identified rice SDH3 subunit (Os02g02940) was poorly related to the SDH3s sequences from Arabidopsis (At5g09600, At4g32210) (Table 1). The short region of similarity contains a conserved His residue that is known from the porcine SDH structure to be the main residue in SDH3 responsible for coordinating heme

binding (Supplemental Fig. 2). The rice genome also contains an additional putative SDH3 isoform (Os07g33680, 70% identical to Os02g02940) (Table 1). A putative SDH4 (Os01g70980) that can be predicted by sequence analysis was also identified as a rice mitochondrial protein by new mass spectrometry data (Table 2). It has only 23% sequence identity with SDH4 in Arabidopsis (At2g46505) in the C-terminal region. This similarity rises to approximately 70% in a small 10 AA region comprising the second His residue involved in heme coordination (Supplemental Fig. 2).

We have previously identified 3 of the plant-specific complex II subunits in rice, namely SDH5, 6, 7 (Huang et al., 2009b) corresponding to the Arabidopsis orthologs identified previously (Eubel et al., 2003; Millar et al., 2004). Here we confirm these identifications by further mass spectrometry (Table 2), but again fail to identify through mass spectrometry or through sequence analysis any rice protein or gene equivalent to the *SDH8* gene in Arabidopsis (At2g46390). It is still possible that rice has a very divergent SDH8 subunit without substantial similarity to Arabidopsis SDH8 yet to be identified.

Expression of genes encoding Complex II subunits in Arabidopsis and rice

To analyse the coordination of gene expression between subunits of Complex II, we extracted the available Arabidopsis and rice microarray data from the NCBI gene expression omnibus (<http://www.ncbi.nlm.nih.gov/geo>) and visualised this in Fig. 1. The detailed information on individual subunits combined with other available data are given below.

At5g66760 (*SDH1-1*) was previously found in all plant tissues with the highest steady-state mRNA levels in flowers and inflorescences, while the At2g18450 (*SDH1-2*) isoform was reported to be only expressed at a very low level (Figueroa et al., 2002). These observations are consistent with the microarray data analysis, with exception of high expression of *SDH1-2* in cultured cells (Fig. 1). Low expression of *SDH1-2* in Arabidopsis might explain why no MS evidence of this gene product has been observed. In rice, expression of Os07g04240 (*SDH1*) was very dependent on the tissues and developmental stage investigated (Fig. 1). Surprisingly Os07G04240 has very low expression in anther tissues (Fig. 1). This was in contrast to our previous finding that genes responsible for TCA cycle are generally highly expressed in rice anther tissues (Huang et al., 2009b). The biochemical regulation of SDH1, the key catalytic subunit for the role of SDH in the TCA cycle will be discussed later based on its biochemical characteristics.

In Arabidopsis, *SDH2-1* and *SDH2-2* (At3g27380 and At5g40650) had similar expression patterns except in the cultured cells (Fig. 1), which were consistent with previous observations that they have similar expression patterns in all organs from adult plants (Figueroa et al., 2001; Elorza et al., 2004). The *SDH2-3* isoform At5g65165, that has not been detected by MS, is also not present on the ATH-1 microarray. However it has been reported to be found to be highly expressed in the embryo during the maturation phase of seed development, and the transcripts are abundant in dry seeds and then decline during germination (Figueroa et al., 2002; Elorza et al., 2006). A recent study revealed that *SDH2-3* had an embryo-specific promoter containing three abscisic acid-responsive elements and one RY-like enhancer element (Roschztardt et al., 2009). In rice, *SDH2-1* (Os08g02640) was highly expressed in most tissues but not in the young leaves and seeds during the late maturing and the early germination stage (Fig. 1). However, the unidentified *SDH2-2* (Os09g20440) was only expressed in the early stage of germination (Fig. 1).

In Arabidopsis and rice, expression of *SDH3*, *SDH4* and *SDH5* are seen to cluster together (Fig. 1). In Arabidopsis, *SDH3* (At4g32210; At5g09600) and *SDH4* (At2g46505) and *SDH5* (At1g47420) appear to be co-expressed with high expression during late germination, in the ovary and embryo (Fig. 1). Similarly, rice *SDH3* (Os02g02940), *SDH4* (Os01g70980), *SDH5* (Os04g34100) and *SDH6* (Os08g02080) show high expression in flowering tissues, late germination and seedling tissues (Fig. 1). The co-expression of *SDH3* and *SDH4* in both species could indicate the requirement of co-ordination for assembly of the membrane components. In Arabidopsis, *SDH6* (At1g08480), *SDH7* (At3g47833 and At5g62575) and *SDH8* (At2g46390) expression is clustered (Fig. 1). In rice, however, *SDH6* (Os08g02080) clustered together with *SDH-3*, *-4* and *-5* (Fig 1). The rice *SDH7* (Os07g21470) was co-expressed with *SDH3* (Os07g33680) and *SDH1* (Os07g04240), protein products for the latter two have not been identified by MS (Fig. 1). Their differential expression patterns are indicative of some common but also some distinctions between Complex II subunits in Arabidopsis and rice. Notably the proteins not found by MS but present in AffyChips have aberrant expression patterns from other subunits of SDH, e.g. *SDH1-2* (At2g18450) in Arabidopsis and *SDH 2-2* (Os09g20440) in rice.

An intact, multi-subunit Complex II can be resolved using Blue-Native (BN) separation in Arabidopsis, but not rice mitochondria.

As Blue Native (BN)-PAGE separation of mitochondrial membrane from dicot plants shows an eight subunit Complex II pattern (Eubel et al., 2003) but the same has not been confirmed in monocots (Heazlewood et al. 2003), we separated rice and Arabidopsis mitochondrial proteins by BN-PAGE and visualised Complex II by activity staining (Eubel et al., 2005). Initially, we followed the original protocol of Eubel et al., (2005) and found only one activity band in Arabidopsis but none in rice. We modified the protocol to give a shorter time run of the first dimension, in order to resolve smaller complexes and found an additional lower band from Arabidopsis and a similar sized band in rice (Fig. 2). Using the modified method, we tested the commonly used detergent digitonin at a series of concentrations to resolve the Arabidopsis and rice membrane proteins (data not shown), we found a ~180 kDa band for complex II from Arabidopsis mitochondria, but only a ~100 kDa band for rice mitochondria (Fig. 2). Mouse mitochondria also contained a ~140 kDa Complex II band, slightly smaller than the Arabidopsis complex but larger than the rice complex (Fig. 2). Further tests using different detergents (Fig. 2) and at different concentrations (data not shown) also failed to generate a higher mass complex in rice mitochondria. To determine if Coomassie was destabilizing complex II in rice, clear-native (CN) gels were also trialled, but there was no high molecular mass complex II activity staining observed in either Arabidopsis or rice (Supplemental Fig 3). This suggested that complex II from plant mitochondria, at least in our hands, is not readily resolved by CN separation, which is different from mammalian complex II which can be resolved by CN (Wittig et al., 2007).

We also conducted second dimension SDS-PAGE gels after separation of mitochondrial proteins dissolved with digitonin (5 g per g protein) by BN PAGE, to investigate the pattern of SDH subunits in Arabidopsis and rice in the region of SDH activity staining (Fig. 3). The apparent protein complex composition for Complex II which aligned with the heavier band in the SDH activity stained gel (Fig. 3), consistent with our previous findings (Eubel et al., 2003; Millar et al., 2004). The rice mitochondrial respiratory complex pattern resolved by BN-PAGE in Fig. 3 was very similar to that previously observed in rice and barley (Eubel et al., 2003; Heazlewood et al., 2003), but no complex II pattern was observed from rice to align with the lighter band in the SDH activity-stained gel lane. In CN-SDS-PAGE gels, there was no complex II pattern analogous to that found in BN-SDS-PAGE gels from Arabidopsis (Supplemental Fig 3). We tried to find SDH subunits in the region marked by the activity stain using mass spectrometry from rice BN gels and BN-SDS-PAGE gels. We found peptides for rice SDH1 as well as matrix proteins such as HSP60 and pyruvate dehydrogenase, but no other SDH subunits (data not

shown). This result suggests that the SDH activity-stained lighter band might belong to a protein subcomplex containing SDH1 but lacking other components like the membrane-anchoring subunits.

ATP activation of succinate-dependent O₂ uptake and SDH enzymatic activity in Arabidopsis is greater than in rice

As outlined above, the activation of mitochondrial SDH by ATP is indirect in mammals, but the evidence in plants from different species is unclear and sometimes contradictory. Recently, we showed that SDH1 (At5g66760) is an ATP binding protein, through an analysis of affinity purification of ATP binding proteins in Arabidopsis mitochondria (Ito et al., 2007), suggesting direct interaction of ATP with an SDH subunit. To help resolve these discrepancies, we investigated activation of mitochondrial SDH by ATP in Arabidopsis and rice by measuring both succinate-dependent oxygen consumption in intact mitochondrial membranes and also activity of the SDH enzyme itself, as succinate-dependent dichlorophenolindophenol (DCPIP) reduction, with or without the use of detergents to solubilise the mitochondrial membrane. The succinate-dependent O₂ consumption rates by isolated Arabidopsis and rice mitochondria (Table 3) were very similar to the rates previously reported (Arabidopsis, 122 ± 19 nmol.min⁻¹ mg⁻¹ prot, Millar et al., 2001; rice, 99 ± 7 nmol.min⁻¹ mg⁻¹ prot, Heazlewood et al., 2003). After addition of 0.5 mM ATP, the O₂ consumption rates increased by only 15% in rice but by nearly 100% in Arabidopsis (Table 3). The malate-dependent O₂ consumption was not significantly increased at all by 0.5 mM ATP in either rice or Arabidopsis (data not shown), indicating the specific activation of succinate-dependent O₂ consumption by exogenous ATP.

To test whether these observed activations could be indirect, for example mediated via the protonmotive force (Affourtit et al., 2001), we conducted in vitro SDH activity assays using freeze-thawed mitochondria or digitonin-treated mitochondria where the membrane was solubilised. The SDH activity was observed to be substantially activated in freeze-thawed mitochondria by addition of ATP. Again, the effect was much greater (6.6-fold) in Arabidopsis than in rice (Table 3, Fig. 4). Furthermore, it took about 2 mins for activation of SDH activity by ATP in Arabidopsis to reach its maximum activity (Fig. 4), which was very similar to the timing of activation of freeze-thawed dicot cauliflower mitochondrial SDH by the addition of 0.5 mM ATP (Oestreicher et al., 1973). In rice, it took approximately 1 min to reach a lower level of ATP activation (Fig. 4). To test whether ATP activation was dependent on membrane potential or not, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to 1 ml reaction solutions to a final

concentration of 4 μ M. The addition of the uncoupler CCCP did not affect the activation of SDH activity by ATP in either plant species (Table 3). In digitonin-treated mitochondria, the activation of SDH by ATP was also observed in both Arabidopsis and rice without any effect by CCCP, but the degree of difference between the responses was much smaller (Table 3). Therefore, we conclude that direct activation of SDH by ATP, independent from membrane potential, can occur in both dicot and monocot mitochondria, but note that the ATP activation phenomenon itself is much less apparent in rice than in Arabidopsis.

Discussion

Complex II is the smallest and perhaps the simplest of all the respiratory electron transport chain complexes, but it plays a central role in mitochondrial metabolism as a member of both the electron transport chain and the tricarboxylic acid (TCA) cycle. Complex II, SDH and the related fumarate reductases have been characterized in bacteria, yeast and mammals, and shown to have a similar four subunit composition (Yankovskaya et al., 2003; Sun et al., 2005). The four classical subunits have been identified in plants by sequence similarity, even without information on complex II structure.

The conservation of SDH1 (flavoprotein) and SDH2 (iron-sulphur protein) in plants was confirmed by the sequence similarity between rice and Arabidopsis subunits (Table 1). SDH1-1 appears to be essential for gametophyte development in Arabidopsis as down-regulation of SDH1-1 by RNA interference results in pollen abortion and a reduced seed set, and homozygous SDH1-1 insertion lines are halted early in gametophyte development (Leon et al., 2007). To date, there has been no available information on the functional analysis of SDH1 in rice.

SDH2-1 and *SDH2-2* genes in Arabidopsis have distinct cell-specific expression patterns and promoter activities, and only *SDH2-2* appears to be expressed at a significant level in root tips (Elorza et al., 2004). The knockout of *SDH2-1* does not have any phenotype, indicating functional redundancy between SDH2-1 and SDH2-2 (Elorza et al., 2004). In rice, there are a more diverse *SDH2* group of genes with a functional chimeric *SDH2-RPS14* encoded by the same gene loci (Os08g02640) for *SDH2* and a related gene (Os09g20440) with a very different 380 amino acid sequence at the N-terminus.

Given that *SDH3* and *SDH4* form not only the membrane anchors but also the UQ binding site and the coordination points for the b type cytochrome, it is surprising that their primary sequences and length can vary so markedly, between the plant and mammal

sequences, and also between plants. One explanation might lie in the recent evolutionary history of *SDH3* and *SDH4* amongst angiosperms. A detailed study of these two small genes has shown that both have been frequently lost from the mitochondrial genome during recent angiosperm evolution and the current nuclear copies in even relatively closely related plant species have independently arisen from gene transfer events (Adams et al., 2001). According to the crystal structure of Complex II in mammals (Sun et al., 2005), the SDH2 subunit strongly interacts with the membrane-anchored subunits through hydrophobic interactions, while there are no points of interaction between SDH1 and SDH3/SDH4. One explanation for the lower conservation in the sequences of SDH2 (Table 1) is as a consequence of modification to fit to the highly variable sequences of SDH3/SDH4 between plants. Alternatively, the complex history of the transfers of *SDH2* and its chimeric import and processing with a ribosomal subunit in monocots, might have influenced the divergence of the sequences of SDH3 and SDH4.

Given the importance of SDH3 and SDH4 in catalysis, the structural integrity of the complex and in its membrane anchoring (Sun et al., 2005), we might expect differences in the stability of Complex II between plants when solubilised by detergents. This might explain why the Arabidopsis complex is intact on BN gels, but the rice complex appears to be disrupted under all the conditions tested here (Fig. 1 and 2). The four additional SDH subunits (SDH5-8) appear to be plant-specific (Eubel et al., 2003; Millar et al., 2004), and while homologs of these Arabidopsis proteins (SDH5-7) can be found in the rice genome, and the proteins have been experimentally found in rice mitochondria (Table 1, 2), we have yet to be able to confirm them as components in rice of a complex containing the classical SDH1-4 subunits.

At the biochemical level, we have shown that the activation of mitochondrial SDH by ATP can occur in both Arabidopsis and rice by a direct route, independent of membrane potential or even of a membrane at all (Table 3). We can interpret the data of Oestreicher et al. (1973); Leach et al. (1995) and Affourtit et al. (2001) in light of our data, as evidence for a divide between monocots and dicots in ATP activation of the SDH enzyme, in much the same way as the sequences of the subunits are divergent, and the stability of the complex in detergents is divergent (Fig. 2 and 3, Eubel et al., 2003). This is not the first time that a clear molecular divide in mitochondrial functions and composition between monocot and dicots has been reported. There is evidence that monocots lack the constitutive type 2 *alternative oxidase* genes responsible for the high alternative oxidase activity of various dicot species (Considine et al., 2002). Also, monocots lack the double lipoyl-domain acetyl transferase (E2) subunits of pyruvate dehydrogenase, but retain the

single lipoyl-domain proteins that are more similar to the yeast E2 enzymes (Millar et al., 1999). But why is SDH directly ATP-activated in plants but not in other organisms? One explanation may lie in the enzyme immediately upstream in the TCA cycle, succinyl-coA ligase. In mammals this enzyme catalyses a substrate-dependent phosphorylation typically of GDP to make GTP, however in plants this is a strictly ADP-dependent enzyme forming ATP (Palmer and Wedding, 1966). Hence in plants, SDH activation by ATP can be viewed as a feed-forward activation of an enzyme by the product of the preceding reaction.

In summary, the systematic analysis of mitochondrial Complex II in Arabidopsis and rice revealed its divergence in sequence, structure and function. The highly variable nature of the SDH3 and SDH4 subunits and the consequent alterations in the complex structure might constitute a primary reason for the differences. As this variability is coincident with the complex genetic history of SDH2, 3 and 4 subunits, this represents a good example of relatively recent coordinated evolution of the subunits of a protein complex critical for aerobic respiration.

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Tables and Figures

Table 1: Similarity between subunits and identification of mitochondrial Complex II subunits in rice and Arabidopsis. The mass spectrometry (MS) evidence for Arabidopsis' mitochondrial Complex II subunits were extracted from the SUBA database (<http://www.plantenergy.uwa.edu.au/applications/suba2>). The MS evidences for rice mitochondrial Complex II were extracted from Huang et al., (2009b). * denotes that Os08g02640 also encodes a chimeric SDH2-RPS14 as shown in Table 2.

Subunit	Arabidopsis	Length (aa)	Rice	Length (aa)	% Identity in region of similarity
SDH1	At5g66760 sdh1-1 (MS) At2g18450 sdh1-2 (No MS)	634 632	Os07g04240 (MS)	630	88% (564/634)
SDH2	At3g27380 sdh2-1 (MS) At5g40650 sdh2-2 (MS) At5g65165 sdh2-3 (No MS)	279 280 309	Os08g02640* (MS) Os09g20440 (No MS)	350 617	70% (193/279)
SDH3	At5g09600 (MS) At4g32210 (No MS)	213 213	Os02g02940 (MS) Os07g33680 (No MS)	129 129	51% (15/29)
SDH4	At2g46505 (MS)	151	Os01g70980 (present 211 study)	211	23% (34/142)
SDH5	At1g47420 (MS)	257	Os04g34100 (MS)	231	58% (100/171)
SDH6	At1g08480 (MS)	142	Os08g02080 (MS)	142	41% (53/127)
SDH7	At3g47833 (MS) At5g62575 (MS)	93 100	Os09g21470 (MS)	90	62% (35/56)
SDH8	At2g46390 (MS)	46	No match	-	-

Table 2. Mass spectrometry identification of Complex II subunits in rice mitochondria using MALDI TOF/TOF MS/MS. MP denotes the number of matched peptides to the protein. Score denotes the total ion score matched to the protein (>36 means $p < 0.05$). Coverage is the percentage of each protein amino acid sequence covered by the identified peptides. The last column defines the transcript-specific peptides (with mascot scores in brackets) confirming the identifications noted for Os08g02640 products. The MS/MS evidence for WIQDSR and ERLDSINDERK are provided in the supplemental Fig 1.

Subunit	Accession Number	Mowse Score	MP	Coverage %	Transcript-specific peptides (ion score)
SDH1	Os07gO4240.1	968	12	27.3	
SDH2	Os08g02640.2	222	6	16.9	WIQDSR (31) ERLDSINDEFK (24)
SDH2-RPS14	Os08g02640.1	187	6	18.5	DPDLPADMQDQFR (58)
SDH3	Os02g02940.1	36	1	10.1	
SDH4	Os01g70980.1	76	2	10.9	
SDH5	Os04g34100.1	497	4	23.8	
SDH6	Os08g02080.1	645	7	48.6	
SDH7	Os09g21470.1	59	1	12.2	

Table 3: Effects of ATP (0.5 mM) on succinate-dependent oxygen consumption of freshly isolated mitochondria and on the enzymatic activities of SDH as succinate-dependent dichlorophenolindophenol (DCPIP) reduction of frozen-thawed mitochondria with or without addition of digitonin (5 g/g protein). The uncoupler CCCP (4 μ M) was also added to test whether the effect of ATP on SDH activity was dependent on the membrane potential or not.

		Rice	Arabidopsis
Succinate-dependent Oxygen consumption		nmol O ₂ min ⁻¹ mg ⁻¹ protein	
	- ATP	98 $\pm$ 14	109 $\pm$ 4
	+ ATP	113 $\pm$ 15	218 $\pm$ 9
	Activation	15%	100 %
Succinate-dependent DCPIP reduction		μ mol DCPIP min ⁻¹ mg ⁻¹ protein	
- Digitonin	- ATP	207 $\pm$ 7	213 $\pm$ 8
	+ ATP	254 $\pm$ 9	533 $\pm$ 25
	Activation	23%	151%
	+ ATP + CCCP	248 $\pm$ 13	530 $\pm$ 29
	Activation	20%	150%
+ Digitonin	- ATP	188 $\pm$ 7	210 $\pm$ 9
	+ ATP	236 $\pm$ 21	405 $\pm$ 30
	Activation	26%	84%
	+ ATP + CCCP	240 $\pm$ 16	384 $\pm$ 42
	Activation	29%	75%

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Fig. 1. Hierarchical clustering of transcripts for genes encoding the subunits of mitochondrial Complex II from Arabidopsis and rice. The Arabidopsis and rice array data was derived from the Gene Expression Omnibus within the National Center of Biotechnology Information (NCBI) database.

Fig 2. Complex II activity stain after one-dimensional Blue Native separation. The mouse, Arabidopsis and rice mitochondrial were solublized with digitonin (5.0 g/g), dodecylmaltoside (DDM, 1.0 g/g), Triton X-100 (1.0 g/g), BigCHAPS (1.0 g/g), CHAPS (1.0 g/g), Tween-20 (1.0 g/g), NP-40 (1.0 g/g).

Fig. 3. Two dimensional resolution of digitonin-solubilized (5 g/g) mitochondrial protein complexes from Arabidopsis (A) and rice (B) by Blue-native/SDS-PAGE. The bottom gels represented the 1-D blue native with Complex II activity staining as described in the Materials and Methods. Designations of the protein complexes are given above the gels. I, NADH dehydrogenase; I+III₂, supercomplex formed by complex I and dimeric complex III; III₂, Dimeric cytochrome c reductase; V, ATP synthase; IV_a + IV_b, large and small form of cytochrome c oxidase.

Fig. 4. The activation of SDH activity of Arabidopsis and rice mitochondria by 0.5 mM ATP (With ATP: solid circle; Without ATP: opened circle). The SDH activity was calculated from supplemental Fig 4. The averages of the maximum activities were given in Table III.

Supplemental Figures

MS/MS Fragmentation of **WIQDSR**

Found in **Os08g02640.2**, 12008.m26455 protein succinate dehydrogenase iron-sulfur protein, mitochondrial precursor, putative, expressed

Monoisotopic mass of neutral peptide Mr(calc): 803.3926

Ions Score: 31 Expect: 0.15

Matches (Bold Red): 15/26 fragment ions using 27 most intense peaks

#	a	a*	b	b*	Seq.	y	y*	#
1	159.0917		187.0866		W			6
2	272.1757		300.1707		I	618.3206	601.2940	5
3	400.2343	383.2078	428.2292	411.2027	Q	505.2365	488.2100	4
4	515.2613	498.2347	543.2562	526.2296	D	377.1779	360.1514	3
5	602.2933	585.2667	630.2882	613.2617	S	262.1510	245.1244	2
6					R	175.1190	158.0924	1

MS/MS Fragmentation of **ERLDSINDEFK**

Found in **Os08g02640.2**, 12008.m26455 protein succinate dehydrogenase iron-sulfur protein, mitochondrial precursor, putative, expressed

Monoisotopic mass of neutral peptide Mr(calc): 1364.6572

Ions Score: 24 Expect: 0.93

Matches (Bold Red): 15/167 fragment ions using 26 most intense peaks

#	Immon.	a	a*	a ⁰	b	b*	b ⁰	Seq.	y	y*	y ⁰	#
1	102.0550	102.0550		84.0444	130.0499		112.0393	E				11
2	129.1135	258.1561	241.1295	240.1455	286.1510	269.1244	268.1404	R	1236.6219	1219.5953	1218.6113	10
3	86.0964	371.2401	354.2136	353.2296	399.2350	382.2085	381.2245	L	1080.5208	1063.4942	1062.5102	9
4	88.0393	486.2671	469.2405	468.2565	514.2620	497.2354	496.2514	D	967.4367	950.4102	949.4262	8
5	60.0444	573.2991	556.2726	555.2885	601.2940	584.2675	583.2835	S	852.4098	835.3832	834.3992	7
6	86.0964	686.3832	669.3566	668.3726	714.3781	697.3515	696.3675	I	765.3777	748.3512	747.3672	6
7	87.0553	800.4261	783.3995	782.4155	828.4210	811.3945	810.4104	N	652.2937	635.2671	634.2831	5
8	88.0393	915.4530	898.4265	897.4425	943.4479	926.4214	925.4374	D	538.2508	521.2242	520.2402	4
9	102.0550	1044.4956	1027.4691	1026.4851	1072.4905	1055.4640	1054.4800	E	423.2238	406.1973	405.2132	3
10	120.0808	1191.5640	1174.5375	1173.5535	1219.5590	1202.5324	1201.5484	F	294.1812	277.1547		2
11	101.1073							K	147.1128	130.0863		1

Supplemental Fig. 1: Mass spectrometry evidence of peptide WIQDSR.and ERLDSINDEFK.

Supplemental Fig 3. Two dimensional resolution of digitonin-solubilized (5 g/g) mitochondrial protein complexes (A) from rice and Arabidopsis by Clear-native/SDS-PAGE. The bottom gels and represented the 1-D clear-native with Complex II activity staining as described in the Materials and Methods. The right panel (B) showed the difference in Complex II activity staining between 1-D blue-native and clear-native gels.

Supplemental Fig 4: Changes of absorbance of DCPIP at 600 nm after adding freeze-thawed Arabidopsis (28 μ g) and rice (25 μ g) mitochondria without or with 0.5 mM ATP in 1 ml reaction solution. The SDH enzymatic activity in Figure 4 was calculated from the slope of curve with combinations of protein content and extinction coefficient of DCPIP.