The mitochondrial LYR protein SDHAF1 is required for succinate dehydrogenase activity in Arabidopsis

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SUMMARY

Succinate dehydrogenase (SDH, complex II), which plays an essential role in mitochondrial respiration and tricarboxylic acid metabolism, requires the assembly of eight nuclear-encoded subunits and the insertion of various cofactors. Here, we report on the characterization of an Arabidopsis thaliana leucine-tyrosine-arginine (LYR) protein family member SDHAF1, (At2g39725) is a factor required for SDH activity. SDHAF1 is located in mitochondria and can fully complement the yeast SDHAF1 deletion strain. Knockdown of SDHAF1 using RNA interference resulted in a decrease in seedling hypocotyl elongation and reduced SDH activity. Proteomic analyses revealed a decreased abundance of various SDH subunits and assembly factors. Protein interaction assays revealed that SDHAF1 can interact exclusively with the Fe-S cluster-containing subunit SDH2 and HSCB, a cochaperone involved in Fe-S cluster complex recruitment. Therefore, we propose that in Arabidopsis, SDHAF1 plays a role in the biogenesis of SDH2 to form the functional complex II, which is essential for mitochondrial respiration and metabolism.

Keywords: mitochondrial metabolism, complex II, succinate dehydrogenase, assembly factors, iron–sulfur cluster insertion.

INTRODUCTION

Plant mitochondria are involved in numerous cellular processes ranging from anabolic and catabolic metabolism to signaling, with the process of oxidative phosphorylation for ATP production dominant in terms of the abundance of the various multisubunit protein complexes involved in this process (Millar et al., 2011). The electron transport chain (ETC) and tricarboxylic acid (TCA) cycle are key mitochondrial processes essential for energy production. The ETC is composed of four complexes (I–IV) on the inner membrane that are involved in electron transfer and proton pumping. Complex II, also known as succinate dehydrogenase (SDH), is an alternative site of electron transfer in the ETC and is also involved in the TCA cycle, oxidizing succinate to fumarate. Complex II is the only mitochondrial enzyme involved in both these major mitochondrial metabolic processes. Complex II consists of four conserved core subunits that contribute to its catalytic function; in Arabidopsis thaliana (Arabidopsis) these are named SDH1, SDH2, SDH3, and SDH4.

The structures of the four core subunits of complex II have been resolved from a number of species such as Escherichia coli, pig (Sus scrofa), and chicken (Gallus domesticus) (Huang et al., 2006; Sun et al., 2005; Yankovskaya et al., 2003). Several complex II subunits contain cofactors required for electron transport. SDH1 contains a flavin adenine dinucleotide (FAD) cofactor at its N-terminus (Sun et al., 2005; Yankovskaya et al., 2003). SDH2 contains three Fe-S clusters, [2Fe-2S], [3Fe-4S], and [4Fe-4S], with [2Fe-2S] attached to its N-terminus and two others that bind to the C-terminus (Sun et al., 2005; Yankovskaya et al., 2003). SDH3 and SDH4 are membrane-anchored proteins that can coordinate heme to form β-type cytochrome (Sun et al., 2005; Yankovskaya et al., 2003).
Whilst the protein sequences of the SDH1 and SDH2 subunits between plants and other organisms are highly conserved, SDH3 and SDH4 show a high degree of sequence divergence across species (Huang et al., 2019; Huang & Millar, 2013). Plant complex II contains four additional subunits, SDH5–8, each encoded by additional genes in plants (Eubel et al., 2003; Huang et al., 2010; Millar et al., 2004). SDH6 and SDH7 may function as replacements for specific helices in SDH3 and SDH4, which are absent in plants but conserved in all other organisms (Schikowsky et al., 2017); the role and origin of SDH5 and SDH8 have not been described.

SDH1 and SDH2, located in the mitochondrial matrix, together form the catalytic SDH enzymatic domain of complex II. This is also the site of electron transfer where electrons from succinate are transferred to FAD and then to Fe-S clusters (Sun et al., 2005; Yankovskaya et al., 2003). In Arabidopsis, two genes encode SDH1, namely SDH1-1 (At5g66760) and SDH1-2 (At2g18450). Knockout of SDH1-1 has been shown to be embryo-lethal and SDH1-2 is expressed at relatively low levels (Figuerola et al., 2002). SDH2 is encoded by three genes, SDH2-1 (At3g27380), SDH2-2 (At5g40650), and SDH2-3 (At5g65165). Arabidopsis SDH2-1 and SDH2-2 are highly expressed in all plant tissues and likely derived from a recent gene duplication event given their 96% protein sequence identity (Figuerola et al., 2001; Elorza et al., 2006). On the other hand, SDH2-3 is only 67% similar to SDH2-1/2 at the protein sequence level and is the only SDH2 present during embryo maturation and germination which suggests it may play a specialized role during desiccation tolerance (Elorza et al., 2006; Heidorn-Czarna et al., 2018; Restovic et al., 2017).

The establishment of a functional complex II requires regulated and coordinated gene expression, cytosolic translation, protein import, and insertion/assembly of its eight individual subunits. Furthermore, complex II can only be fully functional once FAD and Fe-S cofactors are incorporated during the assembly process to convert holoenzyme forms (lacking cofactors) into functional holoenzymes (Kim & Winge, 2013). To date four assembly factors (termed SDHAF1, SDHAF2, SDHAF3, and SDHAF4) have been characterized in mammals, Drosophila melanogaster, and Saccharomyces cerevisiae (Ghezzi et al., 2009; Hao et al., 2009; Maio et al., 2016; Van Vranken Belt et al., 2018; Huang et al., 2013). SDHAF2 was shown to be involved in the insertion of the FAD cofactor within SDH1 (Huang et al., 2013). Knockdown of SDHAF2 in plants caused a decrease in SDH activity, an accumulation of succinate, and reduced abundances of SDH1 and FAD-bound SDH1 (Huang et al., 2013). Knockout of SDHAF4 also resulted in a reduction in SDH activity and accumulation of succinate. In addition, an increased amount of soluble SDH1 was observed in the matrix fraction and a reduced amount of SDH1 was observed in the membrane fraction, indicating that less SDH1 is assembled in the absence of SDHAF4. Therefore, it seems that SDHAF4 acts specifically on the SDH1 subunit to promote its interaction with SDH2 in Arabidopsis (Belt et al., 2018).

Whilst SDHAF1 orthologs have yet to be characterized in any plant species, it is known that yeast and mammalian SDHAF1 are directly involved in the biogenesis of the SDH complex through its interaction with the SDH2 subunit (Maio et al., 2016; Na et al., 2014). SDHAF1 belongs to the mitochondrial LYG protein family, which all contain a conserved leucine-tyrosine-arginine (LYR) domain shown to be required for the recruitment of the Fe-S cluster complex (ISC) to a number of mitochondrial respiratory complex and matrix proteins (Angerer, 2015; Ghezzi et al., 2009). Mutations within the LYG domain of yeast SDHAF1 altered its ability to interact with SDH2, decreased Fe-S insertion into SDH2, and resulted in reduced SDH activity (Maio et al., 2016; Na et al., 2014).

Here, we have characterized the Arabidopsis SDHAF1 ortholog. By combining evidence from yeast complementation and yeast 2-hybrid protein interaction assays, along with the biochemical, metabolic, and phenotypical analysis of SDHAF1 RNA interference (RNAi) lines, we have determined that SDHAF1 is involved in the recruitment of the ISC complex to SDH2 which is otherwise required for normal complex II assembly and activity.

RESULTS

SDHAF1 is a conserved mitochondrial LYG protein

SDHAF1 (At2g39725) has been proposed as a SDHAF1 ortholog in plants based on sequence homology to yeast and human SDHAF1 (Huang et al., 2019; Ivanova et al., 2019). Amino acid sequence alignments of SDHAF1 show an overall sequence similarity of 36–39% with human (Homo sapiens) and yeast (S. cerevisiae) SDHAF1 and the presence of the conserved LYG domain at the N-terminus (Figure 1a). Phylogenetic analysis of SDHAF1 orthologs from various plant species shows its conservation across early plants, monocots, and dicots (Figure 1b). Subcellular localization predictions suggest that SDHAF1 is located in the mitochondria (Hooper et al., 2017). To confirm this localization prediction, SDHAF1 was tagged with GFP at the C-terminus and transiently expressed in Arabidopsis cell culture alongside the mitochondrial marker protein Alternative Oxidase 1a (AOX1a) fused to red fluorescent protein (AOX-RFP) (Carrie et al., 2007) (Figure 1c). A typical mitochondrial pattern of localization was observed as confirmed by the overlay with AOX-RFP, showing that SDHAF1 is localized to the mitochondria (Figure 1c).
SDHAF1 functionally complements yeast SDHAF1 and interacts with SDH subunits and ISC subunits

To investigate if Arabidopsis SDHAF1 (AtSDHAF1) is a functional ortholog to yeast SDHAF1 (ScSDHAF1), yeast complementation assays were carried out (Figure 2a). The yeast SDHAF1 deletion strain (Δsdh6) exhibits a defective growth phenotype when grown on non-fermentative media (Na et al., 2014). Complementation of Δsdh6 with ScSDHAF1 and AtSDHAF1 showed a clear restoration of the growth phenotype when cultured on media containing acetate and glycerol (Figure 2a). This confirms that SDHAF1 (encoded by At2g39725) is a functional ortholog of yeast SDHAF1, able to restore the Δsdh6 defective growth phenotype.

To investigate the ability of Arabidopsis SDHAF1 to interact with subunits of SDH, yeast 2-hybrid interaction assays were carried out. SDHAF1 was cloned into the N- or C-terminally tagged pGADT7 vector and transformed into the AH109 yeast strain. All SDH subunits, SDH1, SDH2, SDH3, SDH4, SDH5, SDH6, SDH7, and SDH8 were cloned into the pGBKT7 vector and transformed into the mating-compatible Y187 strain. In addition, the ISC subunit ISU1 and its cochaperone HSCB (also known as Hsp20) (Leaden et al., 2014; Xu et al., 2009) were cloned into pGBKT7 and all subunits were tested for protein interaction ability with SDHAF1. Transformed yeast was mated and positive interactions were identified by the ability of diploid strains to grown on quadruple drop-out (QDO) selective medium (Figure 2b). All positive interactions identified in three biological replicates were grown and serially diluted on selection media. SDHAF1 was observed to interact with HSCB, SDH2.1, and SDH2.2 (Figure 2b).
proteins SDH1 and SDH2 were observed to interact, as expected, as well as the ISC-interacting proteins HSCB and ISU1 (Leaden et al., 2014; Oyedotun & Lemire, 2004; Sun et al., 2005; Xu et al., 2009) (Figure 2b).

Characterization of Arabidopsis plants disrupted in SDHAF1 expression

To show that SDHAF1 in Arabidopsis is involved in complex II activity and function, as shown for yeast SDHAF1, we sought to obtain or generate Arabidopsis lines defective in SDHAF1. Two independent transfer DNA (T-DNA) insertion lines were initially assessed (Figure S1). One T-DNA insertion line (GABI_694G08) was annotated to have an insertion at the exon region and the second T-DNA insertion line (GABI_536C08) was annotated to have an insertion prior to the 5′ untranslated region. We failed to confirm T-DNA insertion in GABI_694G08 using PCR genotyping with primers as recommended by SALK T-DNA primer design (Figure S1). Nevertheless, these lines were selected for homozygosity based on sulfadiazine resistance and tested for SDHAF1 transcript abundance using quantitative real-time PCR (qRT-PCR). Neither line showed any...
loss in transcript abundance (Figure S1). Furthermore, mitochondria were isolated from GABI_536C08 and no change in SDH activity could be identified; therefore, these lines are not loss-of-function mutants for At2g39725.

As an alternative strategy, an artificial microRNA (amiRNA) approach was undertaken to generate knock-down mutant lines for SDHAF1. Ten individual lines were generated and tested for SDHAF1 transcript abundance. Whilst RNAi-9 exhibited the lowest SDHAF1 expression (16% relative to Col-0) (Figure S2) this line was not viable over multiple generations. Therefore, RNAi-1 and RNAi-10, which both exhibited a substantial decrease in SDHAF1 expression (43% and 28% relative to Col-0, respectively), were selected for further analyses (Figure 3a; Figure S2).

To investigate the phenotypic consequences of SDHAF1 knockdown, we grew RNAi-1 and RNAi-10 lines alongside Col-0 under long-day conditions. We did not observe any obvious phenotypic differences between the two lines and Col-0 (Figure 3b). A comprehensive growth stage progression analysis was carried out during early seedling development that did not reveal any growth defects (Figure S3). Furthermore, various stress conditions were tested, but no growth defects could be identified (Figure S4). As mitochondrial function is vital for energy production in dark-grown seedlings, hypocotyl elongation was tested in dark-grown 14-day-old seedlings in MS medium (Figure 3c). A significant decrease in hypocotyl length was observed in RNAi-1 and RNAi-10, suggesting mitochondrial function was compromised (Student’s t-test, \( P < 0.05, n = 10 \)).

SDHAF1 RNAi lines show a specific decrease in SDH activity and succinate-dependent respiration in isolated mitochondria

To determine whether SDHAF1 knockdown directly affected SDH function, succinate-dependent \( O_2 \) consumption rates were measured on isolated mitochondria from Col-0, RNAi-1, and RNAi-10 lines (Figure 3d(i)). The succinate-dependent \( O_2 \) consumption rates of mitochondria from RNAi-1 and RNAi-10 were reduced to 79% and 64% of Col-0 (Figure 3d(ii)) (Student’s t-test, \( P < 0.05, n = 3 \)). To further confirm a disruption in SDH function, we measured the SDH enzymatic activity in isolated mitochondria using phenazine methosulfate (PMS) and 2,2-dichloro-indophenol (DCPIP) as acceptors (Figure 3d(iii)). SDH activity rates in RNAi-1 and RNAi-10 mitochondria were also significantly reduced to 70% and 56% of Col-0 (Student’s t-test, \( P < 0.05, n = 3 \)) (Figure 3d(iii)). The activities of malate dehydrogenase (MDH), aconitase, and respiratory complex I were not affected in mitochondria isolated from the RNAi lines (Figure 3d).

To investigate if SDH activity and assembly is affected in the mutant lines, we isolated mitochondria and resolved the respiratory chain complexes using Blue native PAGE (BN-PAGE) followed by in-gel activity staining of complex II (Eubel et al., 2005; Schikowsky et al., 2017). While the native 160-kDa band for complex II could not be discerned from a Coomassie-stained gel (Figure 4a, Figure S5), in-gel staining indicated complex II had the same molecular mass in all three lines (Figure 4b, Figure S5). Quantification of in-gel complex II activity indicated that RNAi-10 mitochondria had approximately 50% of the SDH activity measured in Col-0 (Student’s t-test, \( P < 0.05, n = 3 \)) (Figure 4c). A slight decrease in complex I abundance was apparent in RNAi-10 (Figure 4c, Figure S5) yet no significant difference in activity was observed (Figure 3d(v)), which warrants further investigation.

Knockdown of SDHAF1 results in reduced SDH catalytic efficiency

As lowered SDHAF1 expression decreased total SDH activity (Figure 3d), we next investigated the substrate kinetics of SDH activity in both RNAi lines using different concentrations of succinate. A significant decrease in SDH activity was observed in both RNAi lines at succinate concentrations ranging from 0.2 to 10 mM (Figure 5a, Table S5). Maximal enzymatic activity in RNAi-1 and RNAi-10 was lower than in Col-0 (Figure 5b), but there was no difference in the Km of SDH for succinate between Col-0, RNAi-1, and RNAi-10 (Figure 5c). However, catalytic efficiency (Vmax/Km) on a mitochondrial protein basis was calculated to be one-third lower in both RNAi lines (Figure 5d, Table S5), indicating that at the same succinate concentration, the rate of succinate conversion to fumarate was lower in the RNAi lines.

Previously, we reported that succinate accumulates in plant tissues of SDH mutants, including sdhaf2, sdhaf4, and dsr1 (Belt et al., 2018; Gleason et al., 2011; Huang et al., 2013). To test if there were any changes in succinate abundance or associated metabolism in SDHAF1 RNAi lines, we carried out metabolomics analysis. Plants were grown on plates containing MS medium for 10 days under long-day conditions and then leaf and root tissues were collected for analysis. We did not detect any significant difference in succinate content in leaf (Figure 5e) or root tissues in either RNAi line when compared with Col-0 (Figure 5e). The levels of other organic acids, such as fumaric acid, maleic acid, and citric acid, were also unchanged in both RNAi lines compared to Col-0 (Figure 5e.f). However, there were significantly lower abundances of sugars (such as fructose, glucose, galactose, and mannose) in both tissue types of RNAi-10 (Student’s t-test, \( P < 0.05, n = 3 \)) (Figure 5e.f). A summary of the abundance of all detected metabolites is given in Table S1.

Knockdown of SDHAF1 results in reduced abundance of several SDH subunits

The results mentioned above indicate that SDHAF1 knockdown results in decreased SDH activity and succinate-
Succinate dependent O2 uptake (nmolO2 min⁻¹ mg⁻¹ prot)

SDH activity (μmol DCPIP min⁻¹ mg⁻¹ prot)

MDH activity (mM NDAH min⁻¹ mg⁻¹ prot)

Aconitase activity (mM NDAH min⁻¹ mg⁻¹ prot)

Complex I activity (μmol deminco-NADH:FeCN min⁻¹ mg⁻¹ prot)
dependent respiration, but it was not clear if SDHAF1 acted on specific SDH subunits. To investigate the consequences of knockdown of SDHAF1 on individual SDH subunit abundances, protein-specific peptides of SDH subunits and assembly factors were quantified in RNAi-1 and RNAi-10 lines by multiple reaction monitoring (MRM) analysis and differences in abundances were determined (Figure 6, Table S2). The abundance of SDH1, SDH2, and SDH5 was decreased significantly in mitochondrial protein extracts of the RNAi-10 line compared to Col-0 (Figure 6a) (Student’s t-test, $P < 0.05$, $n = 4$). SDH2 was decreased in abundance the most in both lines compared to Col-0 (Figure 6a) (Student’s t-test, $P < 0.05$, $n = 4$), consistent with the hypothesis that SDHAF1 plays a selective role in binding to and stabilizing SDH2. A decreased abundance of SDH2 would change the stoichiometry of SDH2 to SDH1 and may lead to the observed concomitant decrease in SDH1 abundance (Figure 6a). SDH5 is localized at the binding interface of SDH1 and SDH2 in plants (Huang et al., 2019; Schikowsky et al., 2017). It was significantly decreased in abundance in RNAi-10 but not in RNAi-1 (Figure 6a). The assembly factor SDHAF4 was also significantly lower in abundance in RNAi-10 compared to Col-0, while SDHAF2 levels were unchanged (Figure 6a). The more evident changes in RNAi-10 than in RNAi-1 are consistent with the greater severity of the complex II molecular phenotypes in the former (Figure 3). No changes were observed in the abundance of SDH6 and SDH7 subunits (Figure 6a). We could not detect SDHAF1-specific peptides in any of the samples possibly due to their low abundance and the limited set of specific peptide targets available for MRM analysis. To further investigate the abundance of complex II subunits within mitochondrial fractions, MRM analysis was carried out on membrane (pellet) and soluble fractions (supernatant) isolated from mitochondria of RNAi-1 and RNAi-10 lines. In the membrane fraction, the differences in abundance of SDH subunits and assembly factors between the different genotypes were similar to those observed in whole mitochondria (Figure 6b), but significant lower abundances of SDH1 and SDHAF4 could be detected in RNAi-1 which were not previously observed in whole mitochondria samples (Figure 6a). In the soluble fraction, no significant differences in the abundances of any SDH subunits or assembly factors in RNAi-1 or RNAi-10 could be observed. This indicates that the abundance of unassembled SDH1 or SDH2 subunits within the soluble matrix fraction was unchanged, and only the abundance of assembled SDH complex subunits in the membrane was affected by knockdown of SDHAF1 (Figure 6c). The relative peptide abundance ratios of SDH subunits and assembly factors in soluble fraction to membrane fraction were determined, indicating that SDH6 and 7 were mainly membrane-located, while SDHAF2 and 4 were mainly located in the soluble (matrix) fraction (Figure S6).

**DISCUSSION**

Complex II plays a central role in mitochondrial metabolism as a key component of both the ETC and the TCA cycle. The assembly of a fully functional membrane-bound SDH holoenzyme requires several assembly factors for both the insertion of essential cofactors into the holoenzyme and the assembly of various subcomplexes to the membrane-bound holoenzyme (Bezawork-Geleta et al., 2017). SDHAF2 and SDHAF4 have been shown to be involved in the insertion

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**Figure 3.** Transcript abundance of SDHAF1, phenotype, succinate-dependent $O_2$ uptake, and mitochondrial enzymatic activities in SDHAF1 RNAi lines. (a) SDHAF1 expression levels in Col-0, RNAi-1, and RNAi-10 lines. (b) Growth of plants on soil under long-day conditions for 3 weeks. (c) Hypocotyl elongation of dark-grown Col-0, RNAi-1, and RNAi-10 plants on MS medium ($**P < 0.01$, $n = 3$). (d) Succinate-dependent $O_2$ uptake by isolated mitochondria from Col-0, RNAi-1, and RNAi-10. (d i) Malate dehydrogenase (MDH) activity in isolated mitochondria from Col-0, RNAi-1, and RNAi-10. (d ii) Aconitase activity in isolated mitochondria from Col-0, RNAi-1, and RNAi-10. (d iii) Complex I (NADH:ubiquinone oxidoreductase) enzymatic activity in isolated mitochondria from Col-0, RNAi-1, and RNAi-10 ($**P < 0.01$, $n = 3$).

**Figure 4.** BN-PAGE gel separation and complex II in-gel activity staining in SDHAF1 RNAi lines. (a) Coomassie blue staining of BN-PAGE gel. (b) Complex II in-gel activity staining. Complex II is indicated by the white arrow. (c) The relative abundance of activity-stained complex II. $**P < 0.01$, $n = 3$.

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Figure 5. SDH enzymatic kinetics and metabolites in SDHAF1 RNAi lines. (a) SDH activity at succinate concentrations ranging from 0.1 to 10 mM. (b) Kinetic analysis using the Michaelis-Menten formula was performed to determine maximum SDH activity. (c) Km value of SDH for succinate. (d) SDH catalytic efficiency in isolated mitochondria from Col-0 and two RNAi lines. *P < 0.05, **P < 0.01, n = 3. (e) Relative metabolite levels to Col-0 in leaf (left) and root (right) tissues (n = 4). Other detected metabolites are given in Table S1.
of FAD into SDH1 and the interaction between FAD-SDH1 and SDH2 prior to their insertion into the inner membrane-bound complex (Belt et al., 2018; Hao et al., 2009; Huang et al., 2013; Van Vranken et al., 2014). It has been shown in yeast and mammals that SDH assembly requires an additional assembly factor, SDHAF1, for the insertion of Fe-S clusters into SDH2 (Maio et al., 2014; Na et al., 2014). Here, we have characterized the only Arabidopsis SDHAF1 homolog (At2g39725), a mitochondrial LYR domain-containing protein that shares 34% sequence identity with yeast SDHAF1 and was able to fully complement the yeast SDHAF1 deletion strain (Figure 2). Protein interaction assays confirmed the ability of SDHAF1 to interact with its expected target protein SDH2, in addition to the ISC cochaperone HSCB, which has been shown to recruit the ISC to SDH2 (Maio et al., 2014).

In Arabidopsis, knockdown of SDHAF1 using RNAi resulted in decreased SDH enzymatic activity. No impact on the activity of other Fe-S-containing enzymes such as aconitase or complex I could be identified (Figure 3), suggesting that the multiple mitochondrial LYR proteins in this plant species are specific for various components and respiratory chain subunits. Previously, another Arabidopsis LYR protein named Complex I Assembly Factor1 was shown to be specifically involved in the assembly of complex I, and its absence did not affect any other respiratory chain (Ivanova et al., 2019). The complex I defect observed...
in its absence was attributed to the rapid disassembly of specific complex I subunits and submodules compromised in their Fe-S clusters (Ivanova et al., 2021). Knockdown of SDHAF1 reduced the abundance of its expected interacting protein SDH2 and consequently the abundance of SDH2-interacting proteins SDH1 and SDH5 (Figure 6b). The abundance of the complex II in the RNAi-10 mutant line was reduced, as determined by complex II in-gel staining (Figure 4) and based on the decreased rate of conversion of succinate into fumarate by assembled SDH (Figure 3d). BN-PAGE and in-gel staining indicated that SDH complex assembly and mobility in the RNAi-10 mutant line remained comparable to Col-0, suggesting that complex II is correctly assembled, albeit at a lower abundance (Figure 4). This is likely due to the approximately 20–50% decrease of SDHAF1 levels in RNAi-10 compared to Col-0, which still allows for the correct assembly and insertion of SDH subunits within the inner membrane. Additional work would be required to determine if the assembly and/or degradation rates of SDH subunits limit SDH abundance in RNAi-10. An almost complete knockdown of SDHAF1, as observed in RNAi-9, resulted in the rapid loss of seed viability, suggesting that SDHAF1 may be essential for embryogenesis. In addition, SDHAF1 was observed to be expressed in all tissues, with the highest transcript abundance during germination (http://bar.utoronto.ca), a developmental stage with rapid mitochondrial biogenesis (Narsai et al., 2011). Alternatively, it is also possible that the seed viability phenotype is related to the insertion site of the RNAi cassette.

Surprisingly, succinate was not observed to accumulate in SDHAF1 knockdown lines, unlike what has been observed in other SDH enzyme-deficient lines such as dsr1, sdha2, and sdha4 (Belt et al., 2018; Gleason et al., 2011; Huang et al., 2013). It is possible that the reduced SDH activity we observed did not reach the rate-limiting amount necessary for succinate to accumulate. Additionally, the fact that no mutant phenotypes were identified under normal and stress conditions suggests that any consequences to SDH activity are moderate and likely to be compensated for by other mechanisms. Consistently, complete knockout of sdha4 did not alter plant growth and development and sdha2 knockdown only inhibited root elongation, highlighting the compensatory mechanisms that exist to maintain complex II function in plants (Belt et al., 2018; Huang et al., 2013). Deficiency in complex II by knockdown of SDH2 in tomato (Solanum lycopersicum) increased the rate of photosynthesis and growth caused by higher stomatal aperture (Araújo et al., 2011). Similarly, Arabidopsis heterozygous SDH1-1/sdh1-1 plants showed low SDH enzymatic activity but increased photosynthesis rates and improved plant growth when grown under nitrogen-limiting conditions (Fuentes et al., 2011). Thus, targeting of complex II subunits or assembly factors resulting in dysfunction causes different phenotypic variations, consistent with the diversity of physiological functions attributed to SDH (Huang et al., 2019).

SDHAF1 contains a LYR domain, the signature motif of LYR proteins, which has been shown to have diverse functions in Fe-S metabolism and assembly of respiratory chain complexes (Angerer, 2015). In yeast, SDHAF1 was shown to interact with the cochaperone HSCB through its LYR domain (Maio et al., 2014), which is responsible for the subsequent recruitment of the ISC (Maio et al., 2014) to the C-terminus of SDH2 (Maio et al., 2016). Here, we show that SDHAF1 is likely to have the same conserved role in plants, as SDHAF1 was able to interact with SDH2 and the ISC cochaperone HSCB (also known as HSCB) (Figure 2) and its decreased abundance resulted in reduced SDH activity and abundance (Figures 3–6). Therefore, it can be hypothesized that SDHAF1 knockdown is likely to result in altered Fe-S insertion into SDH2, which is required prior to the formation of the SDH1-SDH2 subcomplex (assisted by SDHAF4) that is assembled within the complex (Figure 7). The plant-specific SDH5 subunit has previously been shown to interact with the matrix-localized SDH2 (Schikowsky et al., 2017). Here in the RNAi-10 line we observed a reduction in peptide abundance for both SDH2 and SDH5, which is consistent with this interaction (Figure 6b). However, the levels of subunits SDH6 and SDH7, which have been suggested to serve as replacements for the helices missing from SDH3 and SDH4 (Schikowsky et al., 2017), were not altered. This suggests that the assembly of the matrix SDH1 and SDH2 subunits occurs independently of the membrane-anchored SDH subunits, which is consistent with changes observed in sdhaf4 plants (Belt et al., 2018).

In conclusion, we provide evidence that the function of SDHAF1 is conserved in plants. Arabidopsis SDHAF1 is a mitochondrially located protein that is able to complement the SDHAF1 yeast deletion strain. SDHAF1 knockdown lines exhibit reduced SDH abundance and activity, likely due to the interaction of SDHAF1 with the SDH2 subunit prior to assembly within SDH. SDHAF1 one of the three SDH assembly factor orthologs detected in plant genomes, and the last that remained to be characterized.

**EXPERIMENTAL PROCEDURES**

**Bioinformatic analysis**

Protein sequence information for all SDHAF1 homologs was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozone 12.0 (https://phytozome.jgi.doe.gov/) (Table S3). ClustalW was used to align sequences and MEGA7 (Kumar et al., 2016) was used to generate the tree using the Jones–Taylor–Thornton model and 1000 replications.

**Plant lines and growth conditions**

Arabidopsis (A. thaliana) ecotype Col-0 was used as the control. T-DNA insertion lines (GABI_536C08; GABI_694G08) were ordered from the Nottingham Arabidopsis Stock Centre. Homozygous
Figure 7. A model depicting the function of SDHAF1 in the assembly of the succinate dehydrogenase complex. SDHAF1 interacts with SDH2 and is involved in Fe-S insertion and maturation of SDH2 via an interaction with the ISC cochaperone HSCB. SDH2 assists in FAD insertion within SDH1 (Hao et al., 2009; Huang et al., 2013) and SDHAF4 is subsequently involved in the formation of the SDH1/SDH2 intermediate (Belt et al., 2018; Van Vranken et al., 2014), which is further assembled into the membrane-anchored SDH3/4/5/6 subcomplex. SDHAF1 knockdown results in reduced abundance of SDH1, SDH2, and SDH5 subunits (light green) and consequently reduced activity and abundance of assembled complex II (light green).

plants for T-DNA insertions were selected using PCR-based methods (primers are listed in Table S4 and Figure S1). Seeds were sown on soil containing a 1:3:1 (v/v/v) perlite:shamrock compost: vermiculite mix. Following 3 days of stratification in the dark at 4°C, plants were transferred to a controlled growth chamber (16 h of light/8 h of dark, light intensity 200 μmol m⁻² sec⁻¹, relative humidity 70%, and 22°C day/17°C night). Arabidopsis seeds grown on agar plates were first chlorine gas-sterilized and transferred onto square Petri dishes containing MS medium (half-strength Gamborg B5 vitamin solution, 5 mM MES, 0.8% [w/v] agar, and 2% [w/v] sucrose, pH 5.8). Plates were stored at 4°C for 48 h in the dark and then transferred in a growth chamber with the controlled long-day conditions mentioned above.

**Generation of RNAi lines**

AmiRNA was used to generate RNAi lines for SDHAF1 (A12G39725). The amiRNA designer WMD (wmd3.weigelworld.org) was used to design SDHAF1 amiRNA, which was delivered with a pRS300 plasmid as template for PCRs, containing the mir319a precursor in pBSK. Four oligonucleotide sequences (Table S4) were designed to engineer amiRNA. Precursors for mir319a as backbone for miRNA expression were used under the control of the Cauliflower mosaic virus 35S promoter. In order to exchange the natural miRNA with amiRNA, overlapping PCRs as described in (Schwab et al., 2006) were performed using primers 1 to 4 to replace the natural miRNA. Two additional primers, named A and B (Table S4), were used in a combination of three overlapping PCRs: (1) Primer A and primer 4; (2) primer 3 and primer 2; and (3) primer 1 and primer B. The resulting products were used as a template in a final PCR using primer A and primer B and the product was cloned into pDonor201 (Gateway Technology, Thermo Fisher, Waltham, MA, USA) and recombined into pB2GW7. Arabidopsis plants were transformed using the *Agrobacterium* floral dip method as described previously (Mara et al., 2010).

**Generation of plasmids and constructs**

Gateway technology (Thermo Fisher) was used to generate clones in this study. Full-length cDNA/genomic sequences were amplified by PCR with primers containing attB adapters (attB1 forward primer and attB2 reverse primer; Table S4) and cloned into pDONR201 or pDONR207 using BP Clonase (Thermo Fisher). For some genes, plasmids were obtained from ABRC as entry vectors, pDONR201 or pENTR221. Recombination was carried out using LR Clonase into the destination vectors pDest-CGF (Carrie et al., 2008) for GFP analysis and pAG423_GPD for yeast complementation (from Dr. Susan Lindquist’s lab). Genes encoding SDH subunits were cloned into pGBK7 or pGADT7 using EcoR1- and BamH1-flanked primers. Genes encoding SDHAF1, ISU1, and HSCB were cloned into the Gateway-compatible cg/ng-pGBK77 and cg/ng-pGADT7 (Stellberger et al., 2010) for yeast 2-hybrid (Thermo Fisher) interaction assays.

**Arabidopsis transient transformation using gold particle bombardment**

Transient transformation of Arabidopsis Landsberg cell cultures was carried out using a PDS-1000 gene gun (Bio-Rad, Hercules, CA, USA) as previously described (Duncan et al., 2015). Five micrograms of plasmid DNA coprecipitated onto gold particles was bombarded into 5-day-old cell suspensions using the PDS-1000/He biolistic transformation system (Bio-Rad). GFP and RFP expression was visualized and captured at 100× magnification using an Olympus BX61 microscope at 460/480 nm (GFP) and 570/625 nm (RFP).

**Yeast complementation**

The yeast SDHAF1 deletion strain (Δsdh4, Ydr329c) was ordered from the Saccharomyces Genome Deletion (SGD) Project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). All genes tested were cloned into pAG423_GDP, transformed into yeast, and selected on SD medium lacking histidine (SD-His). Transformants were initially grown in liquid media until reaching confluency in SD-His liquid media with 2% (w/v) glucose, 2% (v/v) glycerol, or 2% (w/v) acetate. The liquid cultures were grown overnight and serially diluted onto SD-His agar plates containing 2% (w/v) glucose, 2% (v/v) glycerol, or 2% (w/v) acetate. Plates were incubated at 30°C and imaged after 3 days.

**Yeast 2-hybrid protein interaction assays**

All bait constructs (pGBK) were transformed into the yeast Y187 haploid strain, whereas all prey constructs (pGAD) were transformed into the yeast AH109 haploid. Yeast were transformed
using the PEG/LiAc method and the dimethyl sulfoxide (DMSO) enhanced heat shock technique (Hill et al., 1991) and selected on SD−/Leu (pGAD) or SD−/Trp (pGBK) media. Yeast mating was performed on flat-bottomed 96-well plates using the Matchmaker GAL4 Two Hybrid System (Clontech, Mountain View, CA, USA) as detailed in the manufacturer’s instructions and yeast were plated onto SD−/Leu−/Trp double drop-out (DDO) medium and SD−/Leu−/−Trp−/Adh−. His QDO medium. Diploid mated strains were identified by growth after 4 days at 30°C on DDO medium whilst strains positive for protein–protein interaction were identified by growth on QDO medium. Positive strains were serially diluted in sterile water and replated onto DDO and QDO media.

**Quantitative RT-PCR to determine SDHAF1 expression**

For transcript analysis Col-0, RNAi-1, and RNAi-10 plants were grown for 3 weeks on soil under long-day conditions. RNA was isolated from rosette leaf tissue using the RNAeasy extraction kit (Qiagen, Hilden, Germany). cDNA synthesis was carried out using the SuperScript III kit (Invitrogen, Waltham, MA, USA). Samples were loaded onto 384-well plates and mixed with 4 µl of SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland). A LightCycler 480 Roche real-time PCR system was used to analyze primers listed in Table S4.

**Isolation of mitochondria from hydroponic cultures**

Arabidopsis seeds were soaked in 70% (v/v) ethanol for 10 min and transferred into sterilization solution (5% (v/v) bleach, 0.1% (v/v) Tween 20) for 10 min. After washing with sterilized water, seeds were transferred into 80 ml of growth medium (half-strength Murashige and Skoog medium with vitamins, half-strength Gamborg B5 vitamin solution, 5 mM MES, 2.5% (w/v) sucrose, pH 5.8) and incubated after shaking (125 rpm) under long-day conditions (16 h light/8 h dark) with a light intensity of 100–125 µmol m−2 sec−1 at 22°C for 2 weeks. Mitochondria were isolated from whole seedlings as outlined previously (De Longevialle et al., 2008) using primers listed in Table S4.

**Measurement of oxygen uptake**

Freshly isolated mitochondria (100 µg each) from Col-0, RNAi-1, and RNAi-10 seedlings were used for oxygen uptake measurements as described previously (Huang et al., 2013) in the presence of 5 mM succinate. Oxygen uptake was analyzed using a Clark-type oxygen electrode (Hansatech Instruments, Pentney, UK).

**SDH activity and kinetics, MDH, aconitase, complex I activities**

All enzymatic activity measurements were performed at 25°C using a spectrophotometer (Shimadzu UV-1800). SDH (complex II), MDH, aconitase, and complex I enzymatic assays were carried out as described previously (Huang et al., 2013). SDH activity was detected using a spectrophotometer via the reduction of DCPIP and measured with a spectrophotometer (Shimadzu UV-1800). SDH (complex II), MDH, aconitase, and complex I enzymatic assays were carried out as described previously (Eubel et al., 2005). Deamin-NADH:Q reductase (complex I) activity was measured in the presence of 5 mM Tris–HCl, pH 7.2, 50 mM NaCl, 1 mM K3[Fe(CN)6], and 0.2 mM deamino-NADH. After adding 10 µg mitochondrial to 1 ml reaction medium, the change of absorbance at 420 nm was measured. The extinction coefficient of K3[Fe(CN)6] at 420 nm is 1.03 mm−1 cm−1.

**BN-PAGE separation and complex II in-gel activity staining**

BN-PAGE was performed out as outlined previously (Eubel et al., 2005) using 5% (w/v) digitonin and precast 4–16% Bis-Tris Gels (NOVEX). To detect complex II in-gel activity, the BN-PAGE gel was stained with 84 mM succinate Na, 0.2 mM phenazine methosulfate, 2 mg ml−1 nitro tetrazolium blue, 4.5 mM EDTA, 10 mM potassium cyanide, and 50 mM potassium phosphate (pH 7.5) buffer for 2–3 h. After staining, the gels were scanned. ImageJ software was used to quantify band intensity.

**Metabolite extraction and gas chromatography–mass spectrometry analysis**

For metabolite analysis, Col-0, RNAi-1, and RNAi-10 plants were grown on MS agar plates for 10 days. Root and rosette leaf tissue was collected in four biological replicates, weighed, and stored at −80°C. Metabolites were extracted using 85% (v/v) methanol in water as outlined previously (Shingaki-Wells et al., 2011). Following derivatization, metabolite samples were analyzed using an Agilent GC/MSD system (Agilent Technologies, Santa Clara, CA, USA). Gas chromatography–mass spectrometry data analysis was carried out using METABOLOME EXPRESS software (version 1.0, http://www.metabolome-express.org) as described previously (Carroll et al., 2010). All data are presented in Table S1.

**MRM sample preparation and analysis**

For MRM analysis, 50 to 100 µg of total mitochondrial, soluble, and membrane fractions from Col-0, RNAi-1, and RNAi-10 was used. To isolate soluble and membrane fractions, whole mitochondrial samples were frozen and thawed three times and vortexed in-between. Centrifugation (20 000 g for 20 min at 4°C) was carried out and the supernatant (soluble fraction) and pellet (membrane fraction) were collected. Samples were acetone-precipitated overnight at −20°C and resuspended in 7 M urea, 2 M thiourea, 50 mM NH4HCO3, and 10 mM DTT at room temperature with shaking at 500 rpm. After the addition of iodoacetamide (25 mM), samples were incubated for 45 min at room temperature in the dark and diluted using 50 mM NH4HCO3 to reach a final urea concentration of less than 1 M. Protein samples were digested with trypsin (0.8 µg µl−1) overnight at 37°C, followed by acidification using 0.1% (v/v) formic acid. After digestion, peptides from

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whole mitochondrial, soluble, and membrane fractions were detected and analyzed using triple-quadrupole mass spectrometry as outlined previously (Huang et al., 2013). The following peptide sequences were selected to quantify protein abundance for SDH subunits or assembly factors: AVIELENGLFSS, SMTMEEV, and SSYTIDHTYADAUGAGAGLGR for SDH1; NEMDPSLTFR for SDH2; VTVLGTGSLGSYVEQR for SDH5; FMEWWR and LSFENN’YTR for SDH6; ALAEADSLR for SDH7; AAAGOPWWR for SDHFA2; and YGDWEQR for SDHFA4. A 6495 triple-quadrupole mass spectrometer (Agilent Technologies) was applied with MassHunter Workstation Data Acquisition software (version B.07.01, build 7.1.712.2; Agilent Technologies). MRM data were analyzed using Skyline (version 3.5.0.9319, MacCoss Laboratory, University of Washington; https://skyline.gs.washington.edu) via integration of peak areas for each quantifier ion. Normalization was performed by comparing the sum of all detected peptides in each run with the sum of ATP synthase peptides (Table S2).

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**AUTHOR CONTRIBUTIONS**

SH and MWM conceived the study and designed the experiments. JW and KB performed most of the experiments. MWM, SH, JW, and AHM analyzed the data and contributed to writing. SA, SS, OVA, and RF carried out experimental procedures. MWM, SH, and JW conceived the study and designed the experiments. YL and KB performed most of the experiments. MWM and AHM wrote the manuscript.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

All relevant data can be found within the manuscript and its supporting materials.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

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