

1 ***Running head:*** SA induced plant stress signalling by mitochondria

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Salicylic acid-dependent plant stress signalling via mitochondrial succinate dehydrogenase

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One sentence summary: Salicylic acid stimulates succinate dehydrogenase activity and induces mitochondrial ROS production to induce stress signalling.

List of author contributions:

AHM, SH, OVA, KB designed the project; KB performed most of the experiments; SH and OVA supervised the experiments; LFT, HC and KBS designed and analysed the luciferase experiments; KB, OVA, SH, AHM performed the writing. All authors contributed to text editing.

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84 **Abstract**

85 Mitochondria are known for their role in ATP production and generation of reactive
86 oxygen species (ROS), but little is known about the mechanism of their early
87 involvement in plant stress signalling. The role of mitochondrial succinate
88 dehydrogenase (SDH) in salicylic acid (SA) signalling was analysed using two
89 mutants; *disrupted in stress response 1* (*dsr1*) which is a point mutation in SDH1
90 identified in a loss of SA signalling screen, and a knockdown mutant (*sdhaf2*) for
91 SDH assembly factor 2 that is required for FAD insertion into SDH1. Both mutants
92 showed strongly decreased SA-inducible stress promoter responses and low SDH
93 maximum capacity compared to wild type; while *dsr1* also showed low succinate
94 affinity, low catalytic efficiency and increased resistance to SDH competitive
95 inhibitors. The SA-induced promoter responses could be partially rescued in *sdhaf2*,
96 but not in *dsr1*, by supplementing the plant growth media with succinate. Kinetic
97 characterisation showed that low concentrations of either SA or ubiquinone binding
98 site inhibitors increased SDH activity and induced mitochondrial H₂O₂ production.
99 Both *dsr1* and *sdhaf2* showed lower rates of SA-dependent H₂O₂ production *in vitro*
100 in line with their low SA-dependent stress signalling responses *in vivo*. This provides
101 quantitative and kinetic evidence that SA acts at or near the ubiquinone binding site
102 of SDH to stimulate activity and contributes to plant stress signalling by increased
103 rates of mitochondrial H₂O₂ production, leading to part of the SA-dependent
104 transcriptional response in plant cells.

Introduction

Within the mitochondrial electron transport chain (mtETC), complex II (Succinate Dehydrogenase: SDH) oxidizes succinate to fumarate by transferring electrons to ubiquinone (UQ), which is reduced to ubiquinol (UQH). The enzyme is formed by four subunits: a flavoprotein (SDH1) which contains the flavin adenine dinucleotide (FAD) cofactor, an iron sulfur (Fe-S) protein (SDH2) housing three Fe-S clusters, and two small integral membrane proteins (SDH3, SDH4), anchoring the enzyme to the inner membrane and forming the ubiquinone binding site (Huang and Millar, 2013, Lemire and Oyedotun, 2002, Sun et al., 2005). Several assembly factors have been identified that facilitate FAD and Fe-S insertion into SDH subunits (Ghezzi et al., 2009, Hao et al., 2009) and one of these, SDHAF2, has been characterised in *Arabidopsis* (Huang et al., 2013).

Complex I and III have been long considered to be the major sources of ROS production inside mitochondria (mtROS), but recent studies in both mammals and plants have demonstrated that complex II can also be a significant source of mtROS (Jardim-Messeder et al., 2015, Quinlan et al., 2012). In mammals, complex II influences reperfusion injury through mtROS production via reverse electron transport after succinate accumulation (Chouchani et al., 2014). However the relative importance of mtROS generated from complex II in plants has been unclear and knockout of the SDH complex or its assembly factors in plants is lethal; largely preventing its direct study through gene deletion in plants (Huang et al., 2013, Leon et al., 2007). This limitation changed when a point mutation of *SDH1-1* (*dsr1*) was identified that did not knockout SDH, but instead lowered SDH activity and decreased mitochondrial ROS production. It was first identified as a mutant that had lost SA- but not H₂O₂-dependent stress response using a *glutathione S-transferase GSTF8* promoter stress response assay (Gleason et al., 2011). The *dsr1* mutant showed steady-state decrease expression of peroxidases, glutaredoxins, and trypsin and protease inhibitor family genes and reduced expression on SA induction of a set of SA-responsive genes normally induced in response to exposure of *Arabidopsis* to bacterial, fungal, or viral pathogens (Gleason et al., 2011). The *dsr1* mutant also had higher susceptibility to fungal and bacterial pathogens indicating that mitochondrial SDH is involved in response to biotic stress *in vivo* in plants. However, despite this evidence for the involvement of a mutated SDH1 and recovery of signalling when

wildtype SDH1 was overexpressed (Gleason et al., 2011), it was still unclear how a mutation in SDH such as *dsr1* could affect mitochondrial ROS production and the downstream stress response induced by SA.

SA acts as a hormone in plant processes like thermogenesis (Raskin et al., 1987), ethylene synthesis and fruit ripening (Leslie and Romani, 1988), but it also acts as a stress regulator during plant defence response (Rao and Davis, 1999, Senaratna et al., 2000, Yalpani et al., 1991). Accumulation of SA is often correlated with an increase in ROS production during plant stress response (as reviewed in Herrera-Vásquez et al. (2015)). A series of SA binding proteins have been identified; notably catalase (Chen et al., 1993a), peroxidase (Durner and Klessig, 1995), and methylsalicylate esterase (Forouhar et al., 2005) that appear to explain this correlation, but their roles as general SA receptors have been controversial (Attaran et al., 2009, Bi et al., 1995). Further sets of SA binding proteins in Arabidopsis have been identified by affinity screens and include several mitochondrial enzymes and also GSTs including GSTF8, which showed enzymatic inhibition by SA (Manohar et al., 2014, Tian et al., 2012). However, as these enzymes are not classical transcription regulators, they are unlikely to directly regulate gene expression. Recently there is clear evidence for NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1, 3 and 4 (NPR1, NPR3 and NPR4) acting together as SA receptors based on their binding properties, direct role in defence gene expression and their impact on disease resistance (Fu et al., 2012, Wu et al., 2012). However, studies beyond defence responses have shown an involvement of SA in thermotolerance and drought resistance combined with an induction of mitochondrial ROS production (Nie et al., 2015, Okuma et al., 2014). SA at high concentration is also reported to act as an inhibitor of respiration in isolated mitochondria but applied in lower concentrations it has been shown to stimulate respiration rate of whole cell tobacco culture (Norman et al., 2004). This indicates the importance of kinetic analysis at an enzymatic level to uncover the role of SA in respiratory responses in plants.

To define the role of SDH in this SA signalling process, we utilised two Arabidopsis mutant lines that have decreased SDH1 function. The fortuitous *dsr1* point mutation acts directly to reduce SDH1 function, while knockdown of an SDH assembly factor (*sdhaf2*) acts indirectly to limit the amount of functional SDH1. We show that both

mutants decrease SA-dependent promoter activity *in vivo*, with *dsr1* more effective than *sdhaf2*. Kinetic analysis of SDH activity in these lines showed that while both mutants had reduced maximum capacity, *dsr1* also differed in succinate affinity and enzymatic efficiency. To determine the nature of the effect of SA and its interaction with SDH for stress signalling, we measured the change in SDH activity in isolated mitochondria in the presence of different concentrations of SA. We observed an SA-dependent increase of SDH activity in the presence of micromolar SA concentrations but only when succinate-dependent electron transport was directed through the UQ binding site of SDH, increasing the succinate:quinone reductase (SQR) activity. We show that succinate-dependent mtROS production increased significantly after the addition of SA in WT, but less so in *dsr1* and *sdhaf2*. *In vivo* we showed that blocking SA-induced promoter activity could be partially relieved in *sdhaf2* by addition of exogenous succinate, but this was not possible with *dsr1*, consistent with our analysis of the differing SDH kinetics in the two mutant lines. Together this provides quantitative and kinetic evidence for a direct involvement of SA in a SDH-dependent signalling pathway in plants that involves mitochondrial ROS production.

Results

Altered stress promoter response to stress in *dsr1* and *sdhaf2*

We previously identified a mutant (*dsr1*), carrying a single *SDH1-1* point mutation, and demonstrated a disruption in SA-induced promoter activity in these plants using a *GSTF8* promoter-driven LUC reporter assay (Gleason et al., 2011). While this effect was linked to SDH1 through a complementation assay, it could not be independently confirmed with knockout plants because loss of SDH1 is embryo lethal in *Arabidopsis* (Huang et al., 2013, Leon et al., 2007). To independently investigate the link between SDH and SA-induced *GSTF8* response, we therefore crossed an SDH assembly factor knockdown line, *sdhaf2*, that has lower SDH activity (Huang et al., 2013) with Col-0 containing the *GSTF8:luc* reporter gene (JC66, referred to as WT in this manuscript; Gleason et al. (2011)). We then treated both mutant lines (*dsr1* and *sdhaf2*) with SA to compare stress promoter response of 4-day-old seedlings (Fig. 1A at 7 mM SA, Supplemental Fig. 1 at 1 mM SA). Both mutants showed low or no responses to the treatment compared to WT (ANOVA

$p \leq 0.01$), however unlike *dsr1*, *sdhaf2* showed significant LUC expression above untreated samples at some time points in the 20 hour period following SA application (Fig. 1A, post-hoc pair-wise test). This strengthened our previous evidence for SDH being involved in SA signalling and showed the effect was independent of the specific amino acid mutation in *dsr1* (Gleason et al., 2011). Both *dsr1* and *sdhaf2* showed a significant LUC expression following H_2O_2 treatment which was not significantly different from WT (Supplemental Fig. 1).

To further confirm that this signalling pathway was SDH-dependent, the SDH inhibitor malonate was added in concentrations of 5 and 10 mM to the growth media and *GSTF8* promoter response was measured after SA treatment. No change in seedling growth and development could be observed in the presence of malonate over a period of 4 days. However, 5 mM malonate could significantly reduce the signal responses in WT and *sdhaf2* (ANOVA $p \leq 0.01$) but the induction of SA signalling in WT was still possible (Fig. 1B, post-hoc pair-wise test). At 10 mM, malonate inhibited the LUC promoter response almost to zero in all genotypes (Fig. 1C). The reduction of stress promoter response, that we observed in both SDH mutant lines and the further inhibition of SDH by treatment with malonate in WT indicate that the degree of function of the SDH enzyme can titrate the degree of stress signalling via this pathway.

Catalytic efficiency of SDH is significantly lower in *dsr1*

To further characterise the *GSTF8* promoter response in *dsr1*, *sdhaf2* and WT, we investigated the kinetics of SDH activity in these lines using PMS and DCPIP (Fig. 2A). We isolated mitochondria from each line and compared the SDH enzymatic catalytic efficiency and substrate affinity using Michaelis-Menten kinetics and Brooks Kinetic Software (Brooks, 1992). In order to calculate the K_m of succinate, a series of succinate concentrations ranging from 0.1 to 10 mM were used for SDH activity measurements (Fig. 2B). Comparing the activity between genotypes over the range of different succinate concentrations, *sdhaf2* and WT shared a similar trend (ANOVA $p = 0.1$), but *dsr1* showed significantly lower activity than WT and *sdhaf2* (ANOVA $p < 0.01$), even when a high concentration of succinate was applied, demonstrating a probable difference in succinate affinity between the two mutants. Looking at the maximum velocity, measured at saturating concentration of succinate (10 mM), there

was a significant distinction in both mutant lines compared to WT (Fig. 2C). It should be noted that in the case of *sdhaf2*, the lower amount of the SDH enzyme (half compared to WT) is responsible for the lower activity rate per mg mitochondria (Huang et al., 2013), whereas in *dsr1* the same amount of SDH enzyme as WT is present in mitochondria (Gleason et al., 2011). Calculation of the K_m value of succinate (Fig. 2D), showed that *dsr1* had a significantly higher K_m than WT and *sdhaf2*. A concentration slightly above 0.4 mM of succinate was required to reach half maximum velocity in WT and *sdhaf2* but over twice as much substrate concentration was needed for *dsr1* (0.86 mM). The catalytic efficiency (V_{max}/K_m), which represents the enzymatic efficiency at low concentrations of substrate, was approximately 3 fold lower in *dsr1* compared to WT and *sdhaf2* (Fig. 2E), showing that *dsr1* was kinetically distinguishable from *sdhaf2*.

***dsr1* shows lower affinity to the competitive inhibitors malonate and OAA**

The changes in SDH kinetics observed in *dsr1* were most likely caused by the point mutation that occurs in the substrate binding site. To further prove that this causes a change in the binding affinity, the competitive inhibitor malonate together with a low concentration (K_m value) of succinate were added to isolated mitochondria from each genotype and SDH activity was measured. Because of the low catalytic efficiency of *dsr1*, twice as much succinate was used in the assay to reach half maximum velocity (0.5 mM for WT and *sdhaf2*, 1 mM for *dsr1*). Using malonate concentrations in a range from 10 to 100 μ M (Fig. 3A top), inhibition of SDH activity was calculated to determine the IC_{50} value for malonate. The inhibition in *dsr1* has less effect on enzyme activity when compared to WT and *sdhaf2*, showing that a higher concentration of inhibitor is necessary to inhibit SDH in *dsr1*. An IC_{50} value of ~ 70 μ M of malonate was determined for *dsr1* compared to a IC_{50} of ~ 20 μ M for WT and *sdhaf2* (Fig. 3B). To confirm that the changes in malonate inhibition were independent of the higher concentration of succinate used in the assay for *dsr1*, the assay was repeated with a saturating (5 mM) concentration of substrate (Supplemental Fig. 2A). A significant inhibition in WT and *sdhaf2* could be reached using 0.1 (WT) and 0.5 mM (*sdhaf2*) malonate. But for *dsr1*, no significant inhibition was caused and SDH was not significantly inhibited even when a concentration of 1 mM was applied. A significant higher IC_{50} of ~ 0.4 mM was calculated for *dsr1* compared to ~ 0.2 mM for *sdhaf2* and WT (Supplemental Fig. 2B). Based on these

kinetic results, we hypothesized that other succinate competitive inhibitors would also show a lower binding affinity in *dsr1*. We applied a second, physiologically more relevant competitive inhibitor, oxaloacetic acid (OAA), together with the same succinate concentrations used in the malonate assay (Fig. 3A bottom) to isolated mitochondria. A significantly higher IC_{50} of 9.6 μ M of OAA for *dsr1* compared to 7 μ M and 6.2 μ M for *sdhaf2* and WT was calculated (Fig. 3B).

Together, these findings demonstrated that the single point mutation in *dsr1* changed the kinetics of SDH and led to a lower binding affinity for the substrate succinate, which results in a lower catalytic efficiency, as well as a lower affinity for the competitive inhibitors malonate and OAA. This is a clear distinction to the knockdown line *sdhaf2*, which has reduced SDH1-1 content (Huang et al., 2013) but does not show any kinetic alterations compared to WT (Fig. 2, 3).

High concentrations of succinate stimulate stress promoter response in *sdhaf2* but not in *dsr1*

Because our data showed that *dsr1* has a low affinity for succinate compared to *sdhaf2* and WT (Fig. 2C, D, E), we investigated if succinate itself would enhance SA-induced signalling. We repeated the *GSTF8:luciferase* assay with 20 mM succinate added to the growth media. No significant induction of promoter activity could be measured in *dsr1* when succinate was present (Fig. 4 bottom), presumably due to its very low catalytic efficiency. However, the promoter activity in *sdhaf2* was significantly induced within 3 hours after the SA treatment in the presence of added succinate (Fig. 4 bottom, post-hoc pairwise test). Because *sdhaf2* shares the same SDH kinetic features as WT, we hypothesized a higher amount of succinate might induce a higher signal response in WT, however the signal was apparently already saturated by the higher SDH enzymatic activity. Nevertheless, we observed a shift in signal response in WT, leading to an earlier peak of signal induction. Higher amounts of succinate might not further increase the signal in WT but could possibly cause a faster response which also declines more rapidly compared to no additional succinate (Fig. 4 bottom).

Low concentrations of SA increases succinate:quinone reductase (SQR) activity

To investigate the role of SA and its interaction with SDH during stress signalling, SQR activity in the presence of SA (10 to 50 μ M) was measured in isolated mitochondria using different electron acceptors. No significant effect of SA was observed for measurements of succinate-dependent DCPIP reduction in the presence of PMS that enables direct acceptance of electrons from the flavin in SDH1 (Fig. 2A, Fig. 5A). However, within SDH, electrons are normally transferred from the succinate binding site in SDH1, through SDH2 and finally to the UQ binding site in the membrane. When the assay was repeated, measuring electron transfer to coenzyme Q₁ and then to DCPIP (Fig. 2A), a significant increase in SQR activity was observed in the presence of SA (Fig. 5B, Supplemental Fig. 3A, Supplemental Table 1). This suggested that the interaction of complex II with SA occurred not at the succinate binding site, but along the electron transfer to UQ or even directly at the UQ binding site. For both mutant lines, a significant increase in electron flow could be measured following SA addition (Supplemental Fig. 3A, Supplemental Table 1), but their overall activity response was lower compared to WT (ANOVA $p < 0.05$). *dsr1* showed the lowest SA induced activity, significantly distinguishable from both *sdhaf2* (ANOVA $p = 0.04$) and WT (ANOVA $p < 0.01$).

Previous studies suggested complex III contained a potential SA binding protein (Nie et al., 2015) and showed inhibition of complex III activity in the presence of 0.1 and 0.5 mM SA. To confirm whether or not complex III activity would be affected by SA, we performed an activity assay using cytochrome c (cyt c) and ubiquinol-10 as substrates and added SA concentrations from 0.01 to 1 mM to the assay (Supplemental Fig. 4). Enzyme activity was determined spectrophotometrically, following the reduction of cyt c. In our hands, no significant differences could be observed in either the genotypes or the response to the SA treatment (Supplemental Fig. 4), confirming that the SA effect observed in this study is Complex II dependent (Fig. 5B).

To further investigate the hypothesis that SA interacts with SDH at the UQ site, compounds known to bind to the UQ site (thenoyltrifluoroacetone (TTFA), carboxin) were added at similar concentrations to SA (Supplemental Fig. 5A, B). SQR activity showed a significant increase in WT in the presence of TTFA and a similar trend was observed in carboxin treatment. Both TTFA and carboxin are commercial complex II

inhibitors with reported IC_{50} of 5.8 μ M and 1.1 μ M in mammals (Miyadera et al., 2003). Nevertheless, using WT Arabidopsis, in our hands, low concentrations of these inhibitors appear to stimulate significantly the electron flow to UQ in a similar manner and at similar concentrations to SA, leading to a faster reduction of DCPIP and a higher SQR activity. Inhibition in Arabidopsis mitochondria was achieved using concentrations of 1 mM TTFA/ carboxin (Supplemental Fig. 5) consistent with other reports in Arabidopsis (Jardim-Messeder et al., 2015, Leon et al., 2007).

To determine if this increased electron transfer to Q_1 in the presence of low concentrations of SA would also be observed via UQ to O_2 in intact mitochondrial electron transport, isolated mitochondria of *sdhaf2* and *dsr1* were treated with SA in the presence of 5 mM succinate and oxygen uptake was measured using a Clark type oxygen electrode. No significant changes in respiration rate across the lines could be observed after adding low concentrations of SA (Fig. 5C, Supplemental Fig. 3B, Supplemental Table 1). Using higher concentrations of SA (0.1 to 1 mM), a gradual inhibition of respiration rate could be observed (Fig. 5D, Supplemental Fig. 3B, Supplemental Table 1), which is consistent with previous studies (Norman et al., 2004). This suggested that enhanced electron transfer from the UQ site to DCPIP in the presence of SA is not observed to significantly increase total respiratory rate in isolated mitochondria ending in the respiratory oxidases.

To test whether other ETC complexes were affected in these genotypes, O_2 uptake in the presence of SA was measured using the substrates NADH, and malate with glutamate (Supplemental Fig. 6A). All genotypes showed sufficient oxygen consumption with these substrates and no significant differences were observed between the mutants and WT. Also no inhibitory effect of SA was observed with either substrate. This confirmed that the decrease in basal respiration observed in *dsr1* and *sdhaf2* (Fig. 5C, D) was specific to succinate and complex II.

Low concentrations of SA induce mitochondrial H_2O_2 production

While respiration rate was not affected by low concentrations of SA, another possibility was, that leakage of electrons occurs at the UQ site, which would result in

partial reduction of oxygen and the formation of reactive oxygen species (ROS) such as O_2^- and H_2O_2 . As ROS production is typically only 3-4% of the total respiratory rate we might not expect to see these changes by monitoring total O_2 consumption (Andreyev et al., 2005, Kudin et al., 2004). To test this hypothesis, freshly isolated mitochondria from plants were treated with SA (0.03 mM) in the presence of 5 mM succinate and 0.5 mM ATP (Fig. 6). We measured succinate-dependent mitochondrial H_2O_2 production using the fluorescent dye DCFDA (Fig. 6). O_2^- has a short lifetime and is a highly reactive molecule that is rapidly converted into H_2O_2 . H_2O_2 is able to leave the mitochondrion (Bienert et al., 2007, Henzler and Steudle, 2000), therefore, the resulting reactive oxygen species that are measured using DCFDA can be assumed to be H_2O_2 . To determine the basal rate of mitochondrial H_2O_2 production, 5 mM succinate and 0.5 mM ATP were added to isolated mitochondria. To determine, if any background fluorescence signal occurred, negative controls for all assays were used (Supplemental Fig. 7). These controls showed that a background signal did occur with just mitochondria and in the absence of respiratory substrate in the sample (Supplemental Fig. 7). Adding SA, in the absence of respiratory substrate to these samples, increased the signal significantly, giving the impression of a high ROS induction, but the actual difference in signal intensity between the plus and minus succinate samples shows that only a small fraction of this signal is succinate-dependent (Supplemental Fig. 7). This fraction was taken as the actual succinate-dependent H_2O_2 production value in our measurements (Fig. 6). Both *dsr1* and *sdhaf2* lines have a lower basal rate of H_2O_2 production when compared with WT (Fig. 6). Antimycin A (AA) was used as a positive control, as it is known to induce production of H_2O_2 (Drose and Brandt, 2008) and we observed a significant increase in H_2O_2 generation when AA was added to mitochondria from all genotypes. To investigate the SA effect on H_2O_2 production, 0.03 mM SA together with succinate and ATP were added to mitochondria. Adding SA caused a significant induction in H_2O_2 production compared to the basal rate (Fig. 6), but the overall rate of H_2O_2 production was still lower in both mutant lines, which showed no significant difference in SA induction compared to the AA treatment.

To test whether other ETC complexes could be a source of SA stimulated ROS production, as was reported in previous studies (Nie et al., 2015), we measured

H₂O₂ production in the presence of NADH and malate together with glutamate (Supplemental Fig. 6B). In our hands, we did not observe any significant ROS production above the background signal without any substrates, as well as no differences between genotypes. Nie et al. (2015) did not use controls in their experiments to show the effects observed were dependent on the presence of respiratory substrates. Their measured signals and SA responses may come from background reactions independent of an active respiratory system inside mitochondria.

Discussion

SDH deficient plants show altered SA-dependent signalling responses

In plants, GSTs are induced by SA, ROS (H₂O₂) and biotic/abiotic stresses (Moons, 2005) and GSTF8 is a well described representative marker for early stress/defense gene induction (Chen et al., 1996, Sappl et al., 2009). In this study we show that the lack of induction of *GSTF8:luciferase* (*GSTF8:luc*) by SA in *dsr1* (Gleason et al., 2011) can be mimicked by reduced FAD insertion and assembly of SDH1-1 through knockdown of the SDH assembly factor SDHAF2. This strengthens the hypothesis that quantitative changes in SDH function are required for at least one pathway of SA induced signalling in plants. The level of promoter activity observed in the *sdhaf2* background was between that of *dsr1* and WT (Fig. 1A, Supplemental Fig. 1 top) demonstrating that the impairment in *sdhaf2* was not completely disabled like it was in *dsr1*, which showed no induction in signal at any time point (Fig. 1A, Supplemental Fig. 1 top). Addition of the SDH competitive inhibitor malonate confirmed that the SA-induced signal is SDH-dependent and that it can be titrated, even in WT (Fig. 1B, C).

Despite general similarities between *dsr1* and *sdhaf2* in promoter activities, the *GSTF8:luc* signal could be partially rescued in *sdhaf2* by the addition of excess succinate, suggesting some different properties of SDH in the two mutants. Kinetic analysis in *dsr1* showed that the SDH enzyme has a significant difference in succinate affinity, catalytic efficiency and inhibition by competitive inhibitors malonate/OAA compared to WT (Fig. 2B, C). This made sense as *dsr1* has a point mutation located at the succinate binding site, which leads to an amino acid change from alanine to threonine (A581T) (Gleason et al., 2011). This change appeared to

cause a lower affinity for succinate and therefore a lower catalytic efficiency in *dsr1* (Fig. 2D, E). Alteration in SDH enzyme kinetics has also been shown in human SDH1 mutations. A point mutation A409C in the succinate binding site of SDH1 led to a 50% reduction of SDH activity and caused optic atrophy and myopathy (Birch-Machin et al., 2000, Sun et al., 2005). Mutation of R554Y in SDH1 caused an unstable SDH1 helix domain and also a 50% decrease in SDH activity and loss of ATP activation resulting in the neurodegenerative disorder Leigh-like syndrome (Bourgeron et al., 1995, Sun et al., 2005). To our knowledge *dsr1* is the first SDH1 mutation shown to alter the K_m of the enzyme for succinate.

These data infer that a certain threshold of SDH activity is required to induce the *GSTF8* SA-dependent promoter stress signal. This activity threshold cannot be reached in *dsr1* and even with higher amounts of succinate no signal induction and no *GSTF8* promoter response occurred (Fig. 4 bottom), leading to pathogen susceptibility (Gleason et al., 2011). This shows that a relatively subtle change in the K_m of a metabolic enzyme can produce a binary switch in stress signalling, raising the possibility that natural variation in metabolic kinetics could be acted upon to improve plant stress sensitivity and tolerance to pathogens. In addition, endogenous inhibitors of SDH like oxaloacetate and malonate act as competitive inhibitors and therefore will change the apparent K_m for succinate, thus acting dynamically in a manner not unlike the *dsr1* mutation, as illustrated by the effect of malonate on WT signalling (Fig. 1B, C).

Low concentrations of SA increase SQR activity

SA is an effective signalling molecule and only micromolar concentrations are required for these effects inside plant cells (Raskin et al., 1987, Wu et al., 2012). The basal level of SA can vary between species and even within the same plant family (Raskin et al., 1990). For *Arabidopsis*, basal levels of SA between 2 μmol to 8 $\mu\text{mol g}^{-1}$ FW have been reported (Brodersen et al., 2005, Klessig et al., 2016, Nawrath and Metraux, 1999, Wildermuth et al., 2001), with SA rising to $\sim 40 \mu\text{mol g}^{-1}\text{FW}$ during infection, which has been equated to approximately 70 μM inside infected plant cells (Bi et al., 1995). The importance of SA in response to biotic and abiotic stress and its involvement in the transcriptional regulation of defense genes has been extensively studied and reviewed (Herrera-Vásquez et al., 2015). Previous

studies of the effect of SA on respiration have focused on the notion of this hormone as an inhibitor and uncoupler of the respiratory chain at concentrations greater than 100 μM (Norman et al., 2004), but no systemic investigations of the effect of low μM levels on respiratory functions have been undertaken. We show here that SA influences the function of complex II at concentrations as low as 10 μM SA when applied to isolated mitochondria (Fig. 5B), potentially placing the effects in the physiological range for Arabidopsis and other SA binding proteins in plants with NPR4 and NRP3 having a SA affinity in nanomolar and micromolar range (Fu et al., 2012, Moreau et al., 2012) as well as several potential effector proteins (catalase, ascorbate peroxidase, carbonic anhydrase) that bind SA with an affinity of 3.7-14 μM (Chen et al., 1993a, Chen et al., 1993b, Durner and Klessig, 1995, Slaymaker et al., 2002).

SA likely interacts with the UQ binding site of complex II

We show the effect of SA on SDH activity did not occur when electrons were accepted directly from SDH1, but only when they were accepted via a quinone. A chemical reaction between SA and the acceptor DCPIP can be excluded as only very low activity was measured when no Q_1 was present in the sample (Fig. 5B), showing that SA together with Q_1 is necessary to allow the induction in activity. This implies that SA does not act via the succinate binding site of SDH1 but instead via or near the UQ binding site of SDH (Fig. 5A, B). We also show that known UQ binding site inhibitors (TTFA, carboxin) can lead to an increase in SQR activity at low micromolar concentrations (Supplemental Fig. 5). TTFA and carboxin are generally described as complex II inhibitors in mammalian and plant system, causing decreased SQR activity and mitochondrial respiration rates at high micromolar to millimolar concentrations (Byun et al., 2008, Jardim-Messeder et al., 2015, Leon et al., 2007, Miyadera et al., 2003, Ramsay et al., 1981). As noted previously, sensitivity of SQR to these inhibitors varies between different species; mammals show a very high sensitivity with IC_{50} values in micromolar concentrations (Miyadera et al., 2003) whereas Arabidopsis SQR is less sensitive, showing inhibitory effects at millimolar concentrations (Supplemental Fig 5; Leon et al. (2007), Jardim-Messeder et al. (2015)). It has also been shown that TTFA binds to a site within SDH3/4 based on X-ray crystallography (Sun et al., 2005). Two binding sites in SDH for quinones have been described for mammals and *E. coli* (Sun et al., 2005, Yankovskaya et al.,

2003). One site (Q_p), located on the matrix side and a second (Q_d) near the intermembrane space site (Hagerhall, 1997). UQ reduction is a single electron two step transfer, forming an ubisemiquinone after the transfer of the first electron, before the complete reduction to ubiquinol occurs following the acceptance of the second electron (Hagerhall, 1997). Inhibitors like TTFA are proposed to block the electron transfer between these two sites, causing electron leakage (Yankovskaya et al., 2003). SA may act similarly to these inhibitors and prevent complete reduction of UQ by blocking the electron transfer from Q_p to Q_d , which could cause electron leakage. Structural similarity between UQ, TTFA and carboxin is not high in strictly chemical terms, but it would appear that SA could structurally mimic some features of both UQ and/or these inhibitors (Supplemental Fig. 8). If SA binds to membrane-embedded SDH3/4 at the UQ binding site as proposed, then this may explain why SDH subunits have not been identified in affinity assay screens for SA binding in Arabidopsis that focused on soluble proteins (Manohar et al., 2014, Tian et al., 2012). Neither the point mutation in *dsr1* nor the assembly defect in *sdhaf2* should affect the UQ site directly, and we did not observe a difference in the SA effect on SQR activity in either line. Although both mutant lines show SA induction, their overall SA induced SQR activity level was still significantly lower than WT and this threshold could be the basis of these mutant effects.

Previous studies have reported complex III as a potential SA binding enzyme (Nie et al., 2015). Within this study, we could not observe any SA effect on complex III activity in any of the lines, neither was there a genotypic difference among the SA treatments (Supplemental Fig. 4). Our results also showed that only when using succinate as substrate, and not when using NADH or malate + glutamate, could SA drive H_2O_2 production above background levels in the absence of respiratory substrates. This strengthens our hypothesis that complex II has a SA binding site near the UQ site and is the major source of H_2O_2 in Arabidopsis mitochondria.

SA stimulates SDH-dependent H_2O_2 production

The effect of SA stimulation of SDH activity in a manner associated with the UQ binding site could lead to reactions with oxygen to form reactive oxygen species (ROS) including superoxide (O_2^-). Within mitochondria, superoxide is rapidly

dismutated by MnSOD to form hydrogen peroxide (H_2O_2). Our previous study showed a clear correlation between SA treatment and accumulation of H_2O_2 (Gleason et al., 2011). WT seedlings treated with SA and the H_2O_2 scavenger catalase showed a reduced GSTF8 signal, showing that this signalling pathway is H_2O_2 -dependent (Gleason et al., 2011). We also showed that exogenous H_2O_2 induces GSTF8 response in WT as well as in *sdhaf2* and *dsr1*, indicating that SDH is involved upstream of ROS signalling (Supplemental Fig. 1 bottom). We measured ROS in isolated mitochondria in the presence and absence of SA, together with succinate and ATP, using DCFDA as a fluorescent marker of H_2O_2 (Fig. 6). DCFDA reacts with any ROS but as O_2^- is highly reactive, unstable and non-membrane permeable, H_2O_2 is the reactive oxygen species that dominates DCFDA fluorescence in isolated mitochondria (Bienert and Chaumont, 2014, Huang et al., 2016). Both mutant lines show a lower basal H_2O_2 production rate compared to WT. Micromolar concentrations of SA induced H_2O_2 production in all genotypes, but significantly less in the mutant lines compared to WT (Fig. 6) and not significantly higher compared to AA treatment. Lower H_2O_2 production in both lines can be explained by their decreased SDH activity (Fig. 2B), even when stimulated by SA at the UQ site (Fig. 5B). Due to lower rate of succinate oxidation in *dsr1* and *sdhaf2*, fewer electrons are transferred to the UQ pool, decreasing its redox poise and slowing the rate of side reactions that would lead to superoxide and then H_2O_2 production. It appears that a threshold of SDH activity needs to be reached in order for increased H_2O_2 production to occur. This observation of enzymatic dependency is similar to the threshold we observed in the GSTF8:luc induction by SA (Fig.1, 4; Supplemental Fig.1). Considering that *sdhaf2* compared to *dsr1* showed a higher GSTF8 promoter signal in the presence of exogenous succinate addition, one might expect to measure a higher H_2O_2 production in this line as well, but this could not be observed (Fig. 6). Differences in the mutants downstream of the SA stress signal pathway might occur to explain these observations.

We noted earlier that we observed a significant background signal with DCFDA that is caused by reactions independent of the respiratory substrate (Supplemental Fig. 7). We found it essential to run control samples parallel to the actual samples to exclude background signals (Fig. 6) that might be caused by site reactions in the sample itself or the autofluorescence of other sample components. Previous studies

investigated the effect of SA in mitochondrial ROS production in Arabidopsis and reported a significant ROS induction after SA addition (Jardim-Messeder et al., 2015). However, no negative controls were used to exclude substrate-independent signals which could mean that the actual substrate dependent signal was significantly lower. In another study, H₂O₂ production in isolated mitochondria has been measured in the presence of different SA concentrations and different substrates for complex I and complex II (Nie et al., 2015). A very high induction of H₂O₂ production was shown after SA was added, but this study also lacks a negative control without substrate. Therefore, the scale of the measured signals in these reports might need reconsideration as they could be substrate-independent and might be mainly caused by background signals occurring in both assays.

We did not observe any significant ROS production above background signals when NADH or malate together with glutamate were used as substrates (Supplemental Fig. 6B), showing that firstly, negative controls without any substrate are essential to determine that any significant signal is not independent of mitochondrial respiration and secondly, that succinate together with SA drives enhanced H₂O₂ production. This demonstrates that complex II can act as a major source of ROS production with higher rates than complex I,III or alternative NADH dehydrogenases, a phenomenon that has previously been shown in mammalian mitochondria where SDH was found to produce the highest amounts of ROS (Dedkova et al., 2013, Quinlan et al., 2012, Ralph et al., 2011) and recently in barley roots, where complex II-derived ROS was shown to be the major source of mitochondrial ROS during mercury toxicity (Tamás and Zelinová, 2017).

The interplay between SA and H₂O₂ and which of these molecules acts first in plant defence appears to vary depending on the pathway being examined (Vlot et al., 2009). We have previously shown that GSTF8 regulation is H₂O₂-dependent (Gleason et al. (2011), Supplemental Fig. 1 bottom) and that accumulation of H₂O₂ follows the SA effect and quantitatively depends on the degree of function of the mitochondrial SDH complex. Earlier studies also showed that SA can enhance H₂O₂ production (Shirasu et al., 1997). Recent studies identified GSTF8 as a SA binding protein (Manohar et al., 2014, Tian et al., 2012) but the biological consequences of that interaction and whether it is involved in stress signalling remains unclear. Based

on our data, it does not seem to interact with GSTF8:*luc* signalling as *dsr1* does not show a signal response after SA treatment (Fig. 1, Supplemental Fig. 1 top).

Besides mitochondria, ROS are also produced in the apoplast, chloroplasts and peroxisomes (Herrera-Vásquez et al., 2015, Love et al., 2008, Vlot et al., 2009) under different stress conditions, and the interaction between organelles is important for an efficient stress response (Herrera-Vásquez et al., 2015). Microarray analysis showed that 18 genes were differentially expressed after SA treatment in *dsr1* vs WT (Gleason et al., 2011), showing that SA induces only a selection of plant defence genes via this pathway and notably it does not directly affect the expression of classical NPR1 targets (Gleason et al., 2011). The SDH-dependent SA pathway described here is thus one part of SA signalling in plants that likely operates independently of how SA is perceived via NPR1/3/4 in plants and in parallel to other ROS-linked pathways that depend on SA-binding proteins (Moreau et al., 2012). Finally, our results add to a growing body of work showing the importance of mitochondria in plant stress/defence responses (Huang et al., 2016), at least in part through the increased production of H₂O₂ from mitochondrial respiratory complexes.

Materials and Methods

Growth of Arabidopsis hydroponic plants

Arabidopsis thaliana (Columbia-0) transgenic lines (JC66, called WT throughout the manuscript), *dsr1* and *sdhaf2* mutant seeds were washed in 70% (v/v) ethanol for 2 min and in sterilization solution (5% (v/v) bleach, 0.1% (v/v) Tween 20) for 5 min with periodical shaking. Seeds were washed 5 times in sterile water before being dispensed into 250 ml plastic vessels containing 80 ml of MS media (half-strength Murashige and Skoog medium without vitamins, half-strength Gamborg B5 vitamin solution, 5 mM MES, 2.5% (w/v) sucrose, pH 7). Hydroponic cultures were grown under 16/8-h light/dark period with light intensity of 100–125 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ at 22°C for 2 weeks or continuously in the dark for the DCFDA measurements.

GSTF8:luciferase signalling of Arabidopsis seedlings

4 days old seedlings of WT, *dsr1* and *sdhaf2* (in the JC66 background) were grown on MS media plus luciferin 1/2 MS medium without vitamins, 1% (w/v) sucrose, pH

7.0, 50 μ M luciferin (Biosynth AG)) with without malonate or succinate using 92x16 mm petri dishes as described previously (Gleason et al., 2011). After incubation with 7 mM SA for 40 min, whole plant bioluminescence was captured over 24 hours using a NightShade imager (Berthold Technologies, Bad Wildbad, Germany) with data calculated in average light units (counts/sec) per seedling using IndiGo (v 2.0.3.0) software (Berthold Technologies).

Isolation of mitochondria from hydroponic cultures

Mitochondria were isolated from 2 weeks old hydroponically grown Arabidopsis plants using a previously described method from Millar et al. (2001), with slight modifications. Plant material was homogenized in grinding buffer (0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 1% (w/v) PVP-40, 2 mM EDTA, 10 mM KH_2PO_4 , 1% (w/v) BSA, 20 mM ascorbic acid, pH 7.5) using mortar and pestle for 2 to 5 min, twice. The homogenate was filtered through four layers of Miracloth and centrifuged at 2500 x g for 5 min, the resulting supernatant was then centrifuged at 14,000 x g for 20 min. The resulting pellet was resuspended in sucrose wash medium (0.3 M sucrose, 0.1% [w/v] BSA, 10 mM TES (N-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid], pH 7.5) and carefully layered over 35 ml PVP-40 gradient (30% Percoll, 0 – 4% PVP). The gradient was centrifuged at 40 000 x g for 40 min. The mitochondrial band was collected and washed 3 times in sucrose wash buffer without BSA at 20 000 x g for 20 min.

Measurement of SDH activity and kinetic calculations

SDH activity was measured directly at the subunit *SDH1-1* by succinate dependent DCPIP reduction at 600 nm. Isolated Arabidopsis mitochondria (50 μ g) were used in 1 ml of reaction medium (50 mM potassium phosphate pH 7.4, 0.1 mM EDTA, 0.1% (w/v) BSA, 10 mM potassium cyanide, 0.12 mM dichlorophenolindophenol (DCPIP) and 1.6 mM phenazine methosulfate (PMS)). To calculate SDH activity, an extinction coefficient of 21 $\text{mM}^{-1} \text{cm}^{-1}$ at 600 nm for DCPIP was used. Brooks Kinetic Software and linear Hanes-Plot calculations were used for kinetic calculations. For measurements targeting the UQ binding site of SDH (SQR activity), 80 μ M Coenzyme Q_1 instead of PMS was used in the reaction medium (Miyadera et al., 2003).

Measurement of Complex III activity

The assay was performed as previously described in Petrosillo et al. (2003). Isolated mitochondria (50 µg) were used in a 1 ml reaction mixture containing 3 mM sodium azide, 1.5 µM rotenone, 50 µM cytochrome c and 50 mM phosphate buffer pH 7.2. The reaction was started by the addition of 50 µM ubiquinol Q10. Complex III activity was determined spectrophotometrically at 550 nm following the reduction of cytochrome c and a rate in nmol cyt c/ min/ mg Mit. was calculated using extinction coefficient (E^{mM}) of 28.0 (reduced cyt c).

Measurement of oxygen consumption using an O₂ Clark electrode

Oxygen consumption was measured using an O₂ Clark electrode. Isolated Arabidopsis mitochondria (100 µg) were used and oxygen uptake measured as previously described in Huang et al. (2013) in the presence of either 5 mM succinate, 1 mM NADH or 10 mM malate+ glutamate. To investigate the effect of SA on respiration, concentrations from 0.01- 1 mM were added after the substrate.

Mitochondrial ROS measurements using DCFDA

DCFDA (2', 7' -dichlorofluorescein diacetate), a cell permeant reagent, which is reacting with ROS within the cell, was used. DCFDA is deacetylated by cellular esterases and forms the fluorescent compound 2', 7' -dichlorofluorescein (DCF) once it is oxidized by ROS. DCF can be detected by fluorescence spectroscopy using excitation/emission spectra of 480/520 nm. Freshly isolated mitochondria (10 µg) from hydroponically grown Arabidopsis plants (continuously in the dark), were transferred in 50 µl buffer (0.3 M sucrose, 5 mM KH₂PO₄, 10 mM TES, 10 mM NaCl, 2 mM MgSO₄, 0.1 % (w/v) BSA, pH 7.2). DCFDA was diluted to 10 µM a final volume of 50 µl in the same buffer solution together with the individual substrates. Both solutions were transferred and mixed in a 96 well plate to a final volume of 100 µl. Fluorescence was measured over 10 min and the slope was calculated.

Figure legends

Figure 1: GSTF8:luc induction in *sdhaf2* and *dsr1* after SA treatment compared to WT.

Average of total fluorescence signal generated by each seedling (n= 10) per hour after treatment of 7 mM SA in the presence of 0 mM (A) , 5 mM (B) and 10 mM (C) malonate (mal) in the growth media. Standard error (SEM); Two-factor ANOVA between genotypes ($p \leq 0.01$), post-hoc Tukey test comparing signal induction to time point zero within genotype * $p \leq 0.05$; ** $p \leq 0.01$.

Figure 2: Lower succinate affinity and catalytic efficiency in *dsr1*

Concentrations of 0.1 to 10 mM of succinate were used to calculate maximal SDH activity, measured as absorbance change of DCPIP at 600 nm. K_m was calculated using Hanes-Plot and Brook Kinetics Software. (A) Scheme of SDH showing electron transfer from succinate to UQ binding site. (B) Correlation of SDH activity and succinate concentrations of WT, *sdhaf2* and *dsr1*. (C) Maximal enzyme velocity (V_{max}). (D) Calculated K_m of succinate using Brooks kinetic software. (E) enzymatic efficiency (V_{max}/K_m) for *sdhaf2* and *dsr1*. Standard error (SEM) of 6 biological replicates; Two-factor ANOVA comparing SDH Activity between genotypes (B) $p \leq 0.01$ (*dsr1* compared to WT and *sdhaf2*), Single-factor ANOVA comparing catalytic efficiency and succinate affinity (D, E) between genotypes. Different letters indicate significant differences ($p \leq 0.05$) between genotypes. n.d. not detected

Figure 3: IC_{50} of SDH competitive inhibitors malonate and oxaloacetate are higher in *dsr1*

Inhibition of SDH was measured using increasing amounts of malonate and OAA together with the K_m concentration of succinate (0.5 mM for WT and *sdhaf2*; 1 mM for *dsr1*). IC_{50} was calculated using Brooks Kinetic Software. (A) Percentage inhibition of SDH activity in the presence of malonate and OAA. (B) Calculated IC_{50} of malonate (left) and OAA (right). Standard error (SEM) of 4 biological replicates; Single-factor ANOVA comparing IC_{50} between genotypes. Different letters indicate significant differences $p \leq 0.07$

Figure 4: SA induced *GSTF8* signal can be rescued in *sdhaf2* using high concentrations of succinate

Average of total fluorescence signal generated by each seedling (n= 10) per hour after treatment of 7 mM SA in the presence of 0 (top) and 20 mM succinate (succ, bottom) in the growth media. Error bars: standard error (SEM); post-hoc Tukey test comparing signal induction to time point zero within genotype, *p≤ 0.05; **p≤ 0.01.

Figure 5: Low concentrations of SA increase SQR activity.

(A) SDH activity measured at the succinate binding site (PMS+DCPIP) in the presence of SA. (B) SQR activity measured at UQ binding site (Q₁ (80 μM) +DCPIP) in the presence of SA. As a negative control activity was measured in the absence of Q₁ in WT mitochondria (yellow bars). In both cases SDH activity was measured in μmol DCPIP/ min/ mg Mit. in the presence of 5 mM succinate and SA concentrations ranging from 0.01 to 0.05 mM. (C, D) Succinate dependent oxygen consumption was measured using a Clark type oxygen electrode in the presence of 5 mM succinate and SA concentrations ranging from 0.01 to 1 mM. Standard error (SEM); Fisher Least Significant Difference (LSD) test was used to determine differences (different letters indicate significant differences (for p-values and letter distribution see Supplemental Table 1, Supplemental Fig. 3), p≤ 0.05

Figure 6: mtH₂O₂ production is lower in *dsr1* and *sdhaf2*

mtH₂O₂ production was measured using DCFDA with excitation/emission wavelengths of 490/520 nm. 5 mM succinate, 0.5 mM ATP, 5 μM AA and 0.03 mM SA were added to freshly isolated mitochondria immediately before the measurement. Fluorescence intensity was measured over 10 min and the rate of fluorescence/min was calculated. Standard error (SEM) of 8 biological replicates; Wilcoxon signed rank test between genotypes, different letters indicate significant differences, p≤ 0.05

Supplemental Materials

Supplemental Figure 1: GSTF8:luc induction in the presence of 1 mM SA or H₂O₂

Supplemental Figure 2: Inhibition of competitive inhibitor malonate in the presence of 5 mM succinate.

Supplemental Figure 3: Significant differences in SQR activity and oxygen consumption between genotypes and SA treatment.

Supplemental Figure 4: Complex III activity in the presence of SA

Supplemental Figure 5: TTFA (A) and carboxin (B) increase SQR activity.

Supplemental Figure 6: Complex I and alternative NADH dehydrogenase dependent ROS and oxygen uptake measurements in the presence of SA.

Supplemental Figure 7: Measured background signals for mitochondrial H₂O₂ production in the absence of substrates and effectors.

Supplemental Figure 8: Comparison of structures for TTFA, Carboxin, SA and ubiquinone-1.

Supplemental Table 1: p-values of statistical analysis between genotypes and treatment (Fisher Least Significant Difference (LSD) test)

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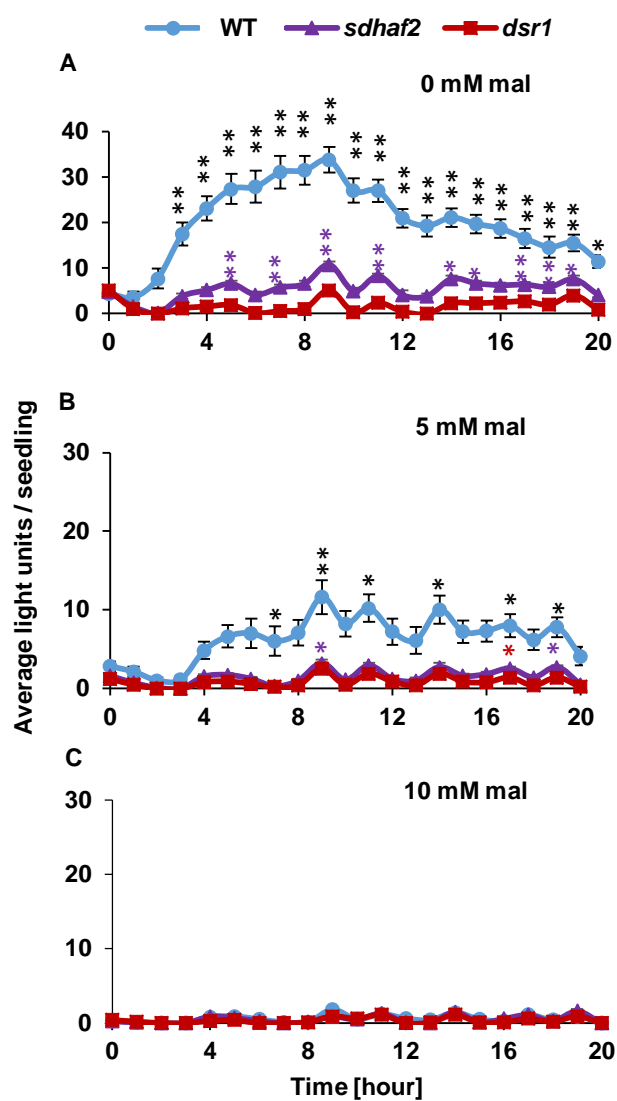
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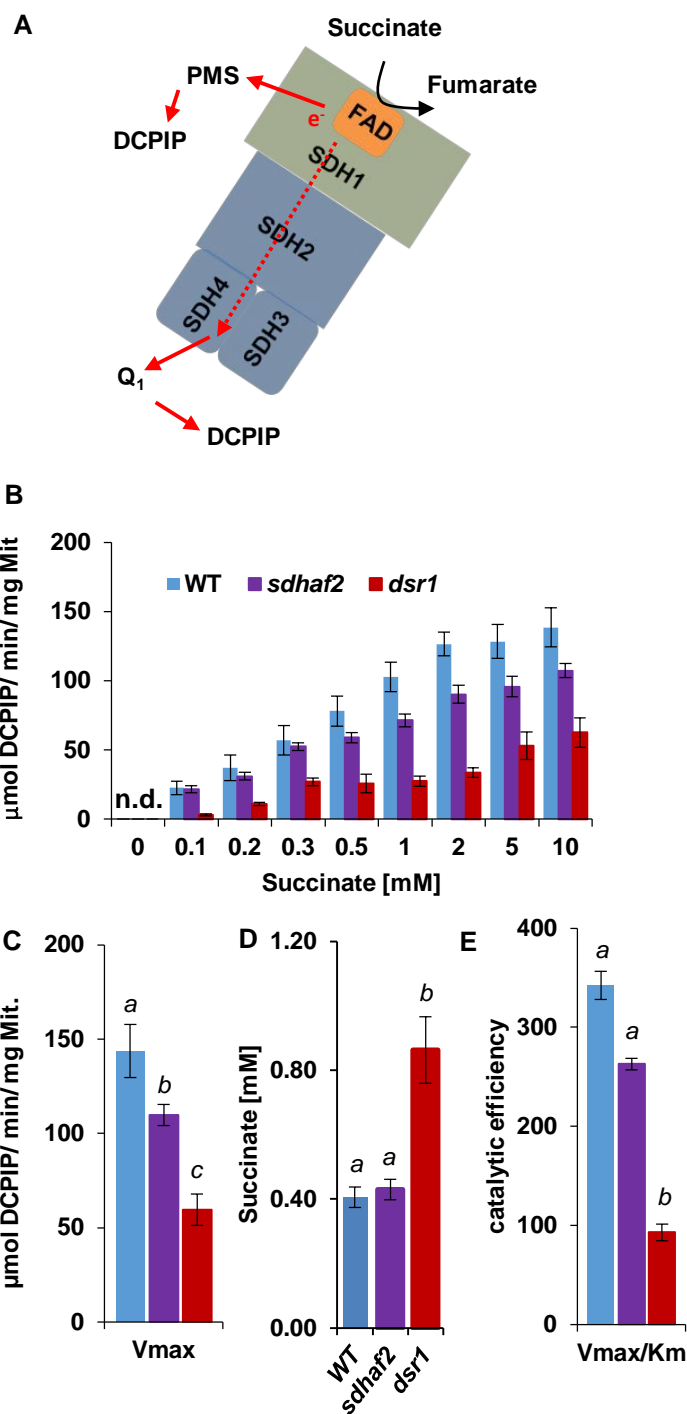
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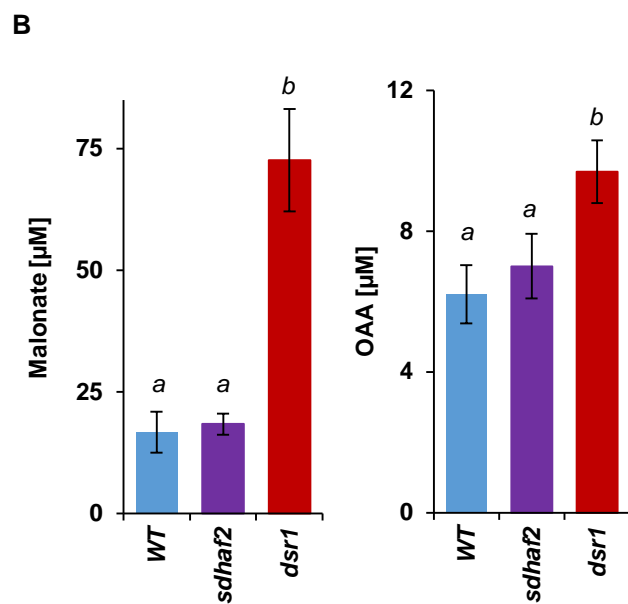
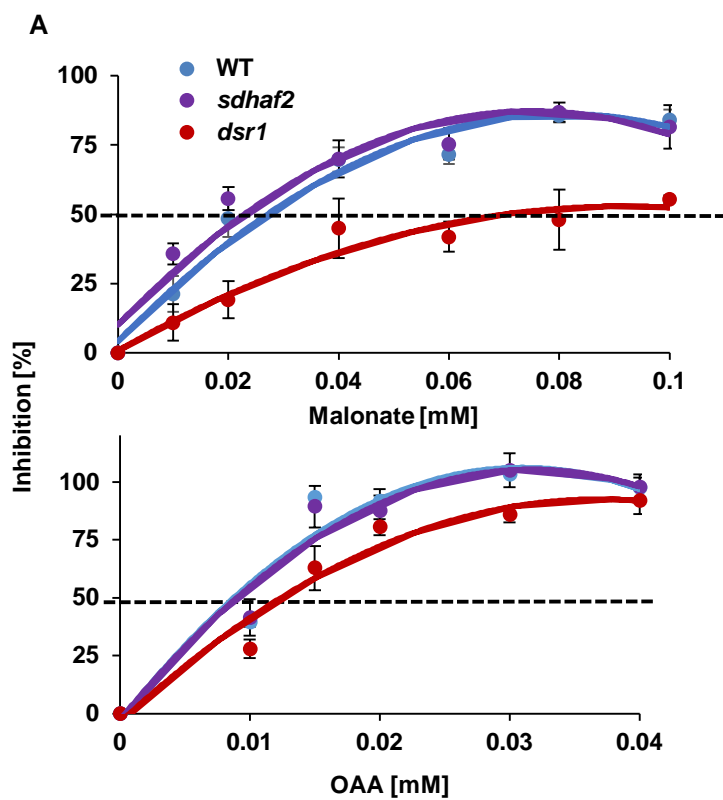
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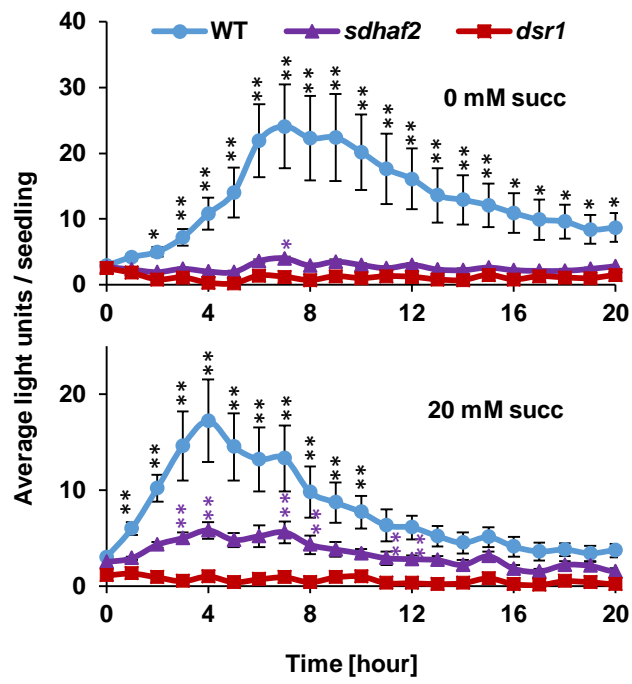
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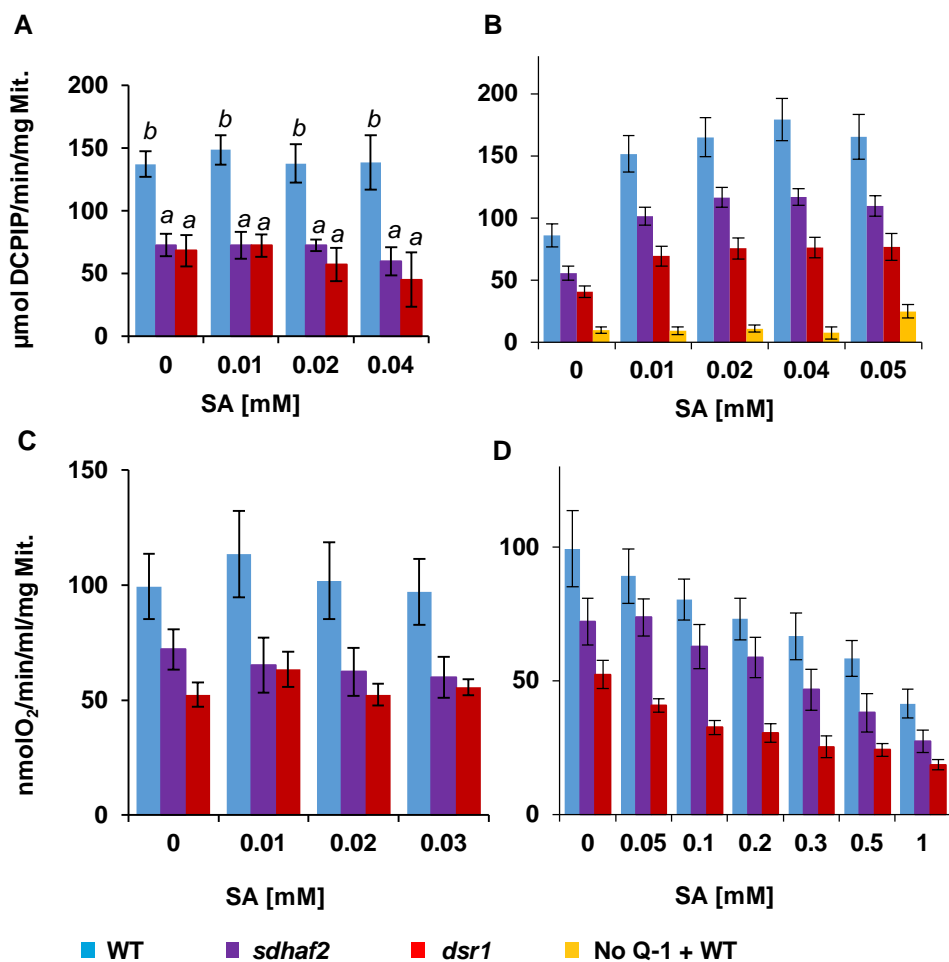
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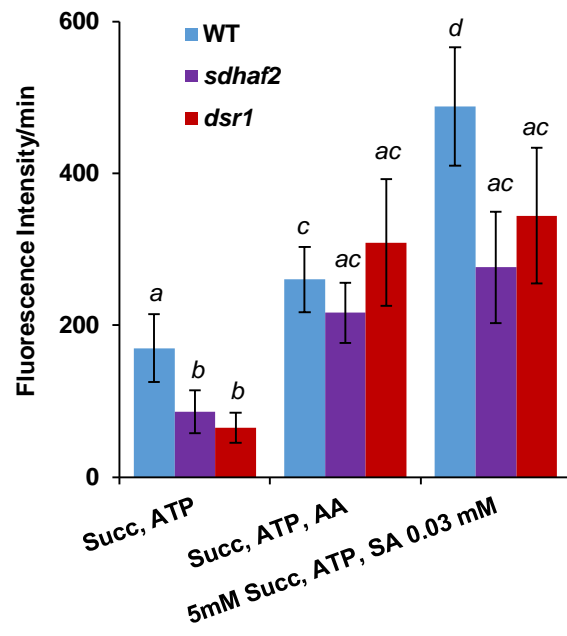












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