

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

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14 Research Area: Mitochondrial Stress Signalling and Response

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26 **Salicylic acid-dependent plant stress signalling via mitochondrial succinate**
27 **dehydrogenase**

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

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44 List of author contributions:

45 AHM, SH, OVA, KB designed the project; KB performed most of the experiments;
46 SH and OVA supervised the experiments; LFT, HC and KBS designed and analysed
47 the luciferase experiments; KB, OVA, SH, AHM performed the writing. All authors
48 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.

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107 **Introduction**

108 Within the mitochondrial electron transport chain (mtETC), complex II (Succinate
109 Dehydrogenase: SDH) oxidizes succinate to fumarate by transferring electrons to
110 ubiquinone (UQ), which is reduced to ubiquinol (UQH). The enzyme is formed by
111 four subunits: a flavoprotein (SDH1) which contains the flavin adenine dinucleotide
112 (FAD) cofactor, an iron sulfur (Fe-S) protein (SDH2) housing three Fe-S clusters,
113 and two small integral membrane proteins (SDH3, SDH4), anchoring the enzyme to
114 the inner membrane and forming the ubiquinone binding site (Huang and Millar,
115 2013, Lemire and Oyedotun, 2002, Sun et al., 2005). Several assembly factors have
116 been identified that facilitate FAD and Fe-S insertion into SDH subunits (Ghezzi et
117 al., 2009, Hao et al., 2009) and one of these, SDHAF2, has been characterised in
118 Arabidopsis (Huang et al., 2013).

119

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

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

144

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

170

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

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

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192

193 **Results**

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

195 We previously identified a mutant (*dsr1*), carrying a single *SDH1-1* point mutation,
196 and demonstrated a disruption in SA-induced promoter activity in these plants using
197 a *GSTF8* promoter-driven LUC reporter assay (Gleason et al., 2011). While this
198 effect was linked to SDH1 through a complementation assay, it could not be
199 independently confirmed with knockout plants because loss of SDH1 is embryo
200 lethal in Arabidopsis (Huang et al., 2013, Leon et al., 2007). To independently
201 investigate the link between SDH and SA-induced *GSTF8* response, we therefore
202 crossed an SDH assembly factor knockdown line, *sdhaf2*, that has lower SDH
203 activity (Huang et al., 2013) with Col-0 containing the *GSTF8:luc* reporter gene
204 (JC66, referred to as WT in this manuscript; Gleason et al. (2011)). We then treated
205 both mutant lines (*dsr1* and *sdhaf2*) with SA to compare stress promoter response of
206 4-day-old seedlings (Fig. 1A at 7 mM SA, Supplemental Fig. 1 at 1 mM SA). Both
207 mutants showed low or no responses to the treatment compared to WT (ANOVA

208 $p \leq 0.01$), however unlike *dsr1*, *sdhaf2* showed significant LUC expression above
209 untreated samples at some time points in the 20 hour period following SA application
210 (Fig. 1A, post-hoc pair-wise test). This strengthened our previous evidence for SDH
211 being involved in SA signalling and showed the effect was independent of the
212 specific amino acid mutation in *dsr1* (Gleason et al., 2011). Both *dsr1* and *sdhaf2*
213 showed a significant LUC expression following H_2O_2 treatment which was not
214 significantly different from WT (Supplemental Fig. 1).

215

216 To further confirm that this signalling pathway was SDH-dependent, the SDH
217 inhibitor malonate was added in concentrations of 5 and 10 mM to the growth media
218 and *GSTF8* promoter response was measured after SA treatment. No change in
219 seedling growth and development could be observed in the presence of malonate
220 over a period of 4 days. However, 5 mM malonate could significantly reduce the
221 signal responses in WT and *sdhaf2* (ANOVA $p \leq 0.01$) but the induction of SA
222 signalling in WT was still possible (Fig. 1B, post-hoc pair-wise test). At 10 mM,
223 malonate inhibited the LUC promoter response almost to zero in all genotypes (Fig.
224 1C). The reduction of stress promoter response, that we observed in both SDH
225 mutant lines and the further inhibition of SDH by treatment with malonate in WT
226 indicate that the degree of function of the SDH enzyme can titrate the degree of
227 stress signalling via this pathway.

228

229 **Catalytic efficiency of SDH is significantly lower in *dsr1***

230 To further characterise the *GSTF8* promoter response in *dsr1*, *sdhaf2* and WT, we
231 investigated the kinetics of SDH activity in these lines using PMS and DCPIP (Fig.
232 2A). We isolated mitochondria from each line and compared the SDH enzymatic
233 catalytic efficiency and substrate affinity using Michaelis-Menten kinetics and Brooks
234 Kinetic Software (Brooks, 1992). In order to calculate the K_m of succinate, a series of
235 succinate concentrations ranging from 0.1 to 10 mM were used for SDH activity
236 measurements (Fig. 2B). Comparing the activity between genotypes over the range
237 of different succinate concentrations, *sdhaf2* and WT shared a similar trend (ANOVA
238 $p = 0.1$), but *dsr1* showed significantly lower activity than WT and *sdhaf2* (ANOVA $p <$
239 0.01), even when a high concentration of succinate was applied, demonstrating a
240 probable difference in succinate affinity between the two mutants. Looking at the
241 maximum velocity, measured at saturating concentration of succinate (10 mM), there

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

254

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

256 The changes in SDH kinetics observed in *dsr1* were most likely caused by the point
257 mutation that occurs in the substrate binding site. To further prove that this causes a
258 change in the binding affinity, the competitive inhibitor malonate together with a low
259 concentration (K_m value) of succinate were added to isolated mitochondria from each
260 genotype and SDH activity was measured. Because of the low catalytic efficiency of
261 *dsr1*, twice as much succinate was used in the assay to reach half maximum velocity
262 (0.5 mM for WT and *sdhaf2*, 1 mM for *dsr1*). Using malonate concentrations in a
263 range from 10 to 100 μ M (Fig. 3A top), inhibition of SDH activity was calculated to
264 determine the IC_{50} value for malonate. The inhibition in *dsr1* has less effect on
265 enzyme activity when compared to WT and *sdhaf2*, showing that a higher
266 concentration of inhibitor is necessary to inhibit SDH in *dsr1*. An IC_{50} value of \sim 70
267 μ M of malonate was determined for *dsr1* compared to a IC_{50} of \sim 20 μ M for WT and
268 *sdhaf2* (Fig. 3B). To confirm that the changes in malonate inhibition were
269 independent of the higher concentration of succinate used in the assay for *dsr1*, the
270 assay was repeated with a saturating (5 mM) concentration of substrate
271 (Supplemental Fig. 2A). A significant inhibition in WT and *sdhaf2* could be reached
272 using 0.1 (WT) and 0.5 mM (*sdhaf2*) malonate. But for *dsr1*, no significant inhibition
273 was caused and SDH was not significantly inhibited even when a concentration of 1
274 mM was applied. A significant higher IC_{50} of \sim 0.4 mM was calculated for *dsr1*
275 compared to \sim 0.2 mM for *sdhaf2* and WT (Supplemental Fig. 2B). Based on these

276 kinetic results, we hypothesized that other succinate competitive inhibitors would
277 also show a lower binding affinity in *dsr1*. We applied a second, physiologically more
278 relevant competitive inhibitor, oxaloacetic acid (OAA), together with the same
279 succinate concentrations used in the malonate assay (Fig. 3A bottom) to isolated
280 mitochondria. A significantly higher IC₅₀ of 9.6 μM of OAA for *dsr1* compared to 7 μM
281 and 6.2 μM for *sdhaf2* and WT was calculated (Fig. 3B).

282

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

289

290 **High concentrations of succinate stimulate stress promoter response in** 291 ***sdhaf2* but not in *dsr1***

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

307

308 **Low concentrations of SA increases succinate:quinone reductase (SQR)** 309 **activity**

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

327

328 Previous studies suggested complex III contained a potential SA binding protein (Nie
329 et al., 2015) and showed inhibition of complex III activity in the presence of 0.1 and
330 0.5 mM SA. To confirm whether or not complex III activity would be affected by SA,
331 we performed an activity assay using cytochrome c (cyt c) and ubiquinol-10 as
332 substrates and added SA concentrations from 0.01 to 1 mM to the assay
333 (Supplemental Fig. 4). Enzyme activity was determined spectrophotometrically,
334 following the reduction of cyt c. In our hands, no significant differences could be
335 observed in either the genotypes or the response to the SA treatment (Supplemental
336 Fig. 4), confirming that the SA effect observed in this study is Complex II dependent
337 (Fig. 5B).

338

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

344 inhibitors with reported IC_{50} of 5.8 μ M and 1.1 μ M in mammals (Miyadera et al.,
345 2003). Nevertheless, using WT Arabidopsis, in our hands, low concentrations of
346 these inhibitors appear to stimulate significantly the electron flow to UQ in a similar
347 manner and at similar concentrations to SA, leading to a faster reduction of DCPIP
348 and a higher SQR activity. Inhibition in Arabidopsis mitochondria was achieved using
349 concentrations of 1 mM TTFA/ carboxin (Supplemental Fig. 5) consistent with other
350 reports in Arabidopsis (Jardim-Messeder et al., 2015, Leon et al., 2007).

351

352 To determine if this increased electron transfer to Q_1 in the presence of low
353 concentrations of SA would also be observed via UQ to O_2 in intact mitochondrial
354 electron transport, isolated mitochondria of *sdhaf2* and *dsr1* were treated with SA in
355 the presence of 5 mM succinate and oxygen uptake was measured using a Clark
356 type oxygen electrode. No significant changes in respiration rate across the lines
357 could be observed after adding low concentrations of SA (Fig. 5C, Supplemental Fig.
358 3B, Supplemental Table 1). Using higher concentrations of SA (0.1 to 1 mM), a
359 gradual inhibition of respiration rate could be observed (Fig. 5D, Supplemental Fig.
360 3B, Supplemental Table 1), which is consistent with previous studies (Norman et al.,
361 2004). This suggested that enhanced electron transfer from the UQ site to DCPIP in
362 the presence of SA is not observed to significantly increase total respiratory rate in
363 isolated mitochondria ending in the respiratory oxidases.

364

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

372

373

374 **Low concentrations of SA induce mitochondrial H_2O_2 production**

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

377 partial reduction of oxygen and the formation of reactive oxygen species (ROS) such
378 as O_2^- and H_2O_2 . As ROS production is typically only 3-4% of the total respiratory
379 rate we might not expect to see these changes by monitoring total O_2 consumption
380 (Andreyev et al., 2005, Kudin et al., 2004). To test this hypothesis, freshly isolated
381 mitochondria from plants were treated with SA (0.03 mM) in the presence of 5 mM
382 succinate and 0.5 mM ATP (Fig. 6). We measured succinate-dependent
383 mitochondrial H_2O_2 production using the fluorescent dye DCFDA (Fig. 6). O_2^- has a
384 short lifetime and is a highly reactive molecule that is rapidly converted into H_2O_2 .
385 H_2O_2 is able to leave the mitochondrion (Bienert et al., 2007, Henzler and Steudle,
386 2000), therefore, the resulting reactive oxygen species that are measured using
387 DCFDA can be assumed to be H_2O_2 . To determine the basal rate of mitochondrial
388 H_2O_2 production, 5 mM succinate and 0.5 mM ATP were added to isolated
389 mitochondria. To determine, if any background fluorescence signal occurred,
390 negative controls for all assays were used (Supplemental Fig. 7). These controls
391 showed that a background signal did occur with just mitochondria and in the absence
392 of respiratory substrate in the sample (Supplemental Fig. 7). Adding SA, in the
393 absence of respiratory substrate to these samples, increased the signal significantly,
394 giving the impression of a high ROS induction, but the actual difference in signal
395 intensity between the plus and minus succinate samples shows that only a small
396 fraction of this signal is succinate-dependent (Supplemental Fig.7). This fraction was
397 taken as the actual succinate-dependent H_2O_2 production value in our
398 measurements (Fig. 6). Both *dsr1* and *sdhaf2* lines have a lower basal rate of H_2O_2
399 production when compared with WT (Fig. 6). Antimycin A (AA) was used as a
400 positive control, as it is known to induce production of H_2O_2 (Drose and Brandt,
401 2008) and we observed a significant increase in H_2O_2 generation when AA was
402 added to mitochondria from all genotypes. To investigate the SA effect on H_2O_2
403 production, 0.03 mM SA together with succinate and ATP were added to
404 mitochondria. Adding SA caused a significant induction in H_2O_2 production compared
405 to the basal rate (Fig. 6), but the overall rate of H_2O_2 production was still lower in
406 both mutant lines, which showed no significant difference in SA induction compared
407 to the AA treatment.

408

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

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

419

420 **Discussion**

421

422 **SDH deficient plants show altered SA-dependent signalling responses**

423 In plants, GSTs are induced by SA, ROS (H₂O₂) and biotic/abiotic stresses (Moons,
424 2005) and GSTF8 is a well described representative marker for early stress/defense
425 gene induction (Chen et al., 1996, Sappl et al., 2009). In this study we show that the
426 lack of induction of *GSTF8:luciferase* (*GSTF8:luc*) by SA in *dsr1* (Gleason et al.,
427 2011) can be mimicked by reduced FAD insertion and assembly of SDH1-1 through
428 knockdown of the SDH assembly factor SDHAF2. This strengthens the hypothesis
429 that quantitative changes in SDH function are required for at least one pathway of
430 SA induced signalling in plants. The level of promoter activity observed in the *sdhaf2*
431 background was between that of *dsr1* and WT (Fig. 1A, Supplemental Fig. 1 top)
432 demonstrating that the impairment in *sdhaf2* was not completely disabled like it was
433 in *dsr1*, which showed no induction in signal at any time point (Fig. 1A, Supplemental
434 Fig. 1 top). Addition of the SDH competitive inhibitor malonate confirmed that the SA-
435 induced signal is SDH-dependent and that it can be titrated, even in WT (Fig. 1B, C).

436

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

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

454

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

467

468 **Low concentrations of SA increase SQR activity**

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

479 studies of the effect of SA on respiration have focused on the notion of this hormone
480 as an inhibitor and uncoupler of the respiratory chain at concentrations greater than
481 100 μM (Norman et al., 2004), but no systemic investigations of the effect of low μM
482 levels on respiratory functions have been undertaken. We show here that SA
483 influences the function of complex II at concentrations as low as 10 μM SA when
484 applied to isolated mitochondria (Fig. 5B), potentially placing the effects in the
485 physiological range for Arabidopsis and other SA binding proteins in plants with
486 NPR4 and NRP3 having a SA affinity in nanomolar and micromolar range (Fu et al.,
487 2012, Moreau et al., 2012) as well as several potential effector proteins (catalase,
488 ascorbate peroxidase, carbonic anhydrase) that bind SA with an affinity of 3.7-14 μM
489 (Chen et al., 1993a, Chen et al., 1993b, Durner and Klessig, 1995, Slaymaker et al.,
490 2002).

491

492 **SA likely interacts with the UQ binding site of complex II**

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

513 2003). One site (Q_p), located on the matrix side and a second (Q_d) near the
514 intermembrane space site (Hagerhall, 1997). UQ reduction is a single electron two
515 step transfer, forming an ubisemiquinone after the transfer of the first electron,
516 before the complete reduction to ubiquinol occurs following the acceptance of the
517 second electron (Hagerhall, 1997). Inhibitors like TTFA are proposed to block the
518 electron transfer between these two sites, causing electron leakage (Yankovskaya et
519 al., 2003). SA may act similarly to these inhibitors and prevent complete reduction of
520 UQ by blocking the electron transfer from Q_p to Q_d , which could cause electron
521 leakage. Structural similarity between UQ, TTFA and carboxin is not high in strictly
522 chemical terms, but it would appear that SA could structurally mimic some features
523 of both UQ and/or these inhibitors (Supplemental Fig. 8). If SA binds to membrane-
524 embedded SDH3/4 at the UQ binding site as proposed, then this may explain why
525 SDH subunits have not been identified in affinity assay screens for SA binding in
526 Arabidopsis that focused on soluble proteins (Manohar et al., 2014, Tian et al.,
527 2012). Neither the point mutation in *dsr1* nor the assembly defect in *sdhaf2* should
528 affect the UQ site directly, and we did not observe a difference in the SA effect on
529 SQR activity in either line. Although both mutant lines show SA induction, their
530 overall SA induced SQR activity level was still significantly lower than WT and this
531 threshold could be the basis of these mutant effects.

532

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

541

542

543 **SA stimulates SDH-dependent H_2O_2 production**

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

547 dismutated by MnSOD to form hydrogen peroxide (H_2O_2). Our previous study
548 showed a clear correlation between SA treatment and accumulation of H_2O_2
549 (Gleason et al., 2011). WT seedlings treated with SA and the H_2O_2 scavenger
550 catalase showed a reduced *GSTF8* signal, showing that this signalling pathway is
551 H_2O_2 -dependent (Gleason et al., 2011). We also showed that exogenous H_2O_2
552 induces *GSTF8* response in WT as well as in *sdhaf2* and *dsr1*, indicating that SDH is
553 involved upstream of ROS signalling (Supplemental Fig. 1 bottom). We measured
554 ROS in isolated mitochondria in the presence and absence of SA, together with
555 succinate and ATP, using DCFDA as a fluorescent marker of H_2O_2 (Fig. 6). DCFDA
556 reacts with any ROS but as O_2^- is highly reactive, unstable and non-membrane
557 permeable, H_2O_2 is the reactive oxygen species that dominates DCFDA fluorescence
558 in isolated mitochondria (Bienert and Chaumont, 2014, Huang et al., 2016). Both
559 mutant lines show a lower basal H_2O_2 production rate compared to WT. Micromolar
560 concentrations of SA induced H_2O_2 production in all genotypes, but significantly less
561 in the mutant lines compared to WT (Fig. 6) and not significantly higher compared to
562 AA treatment. Lower H_2O_2 production in both lines can be explained by their
563 decreased SDH activity (Fig. 2B), even when stimulated by SA at the UQ site (Fig.
564 5B). Due to lower rate of succinate oxidation in *dsr1* and *sdhaf2*, fewer electrons are
565 transferred to the UQ pool, decreasing its redox poise and slowing the rate of side
566 reactions that would lead to superoxide and then H_2O_2 production. It appears that a
567 threshold of SDH activity needs to be reached in order for increased H_2O_2 production
568 to occur. This observation of enzymatic dependency is similar to the threshold we
569 observed in the *GSTF8:luc* induction by SA (Fig.1, 4; Supplemental Fig.1).
570 Considering that *sdhaf2* compared to *dsr1* showed a higher *GSTF8* promoter signal
571 in the presence of exogenous succinate addition, one might expect to measure a
572 higher H_2O_2 production in this line as well, but this could not be observed (Fig. 6).
573 Differences in the mutants downstream of the SA stress signal pathway might occur
574 to explain these observations.

575

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

581 investigated the effect of SA in mitochondrial ROS production in Arabidopsis and
582 reported a significant ROS induction after SA addition (Jardim-Messeder et al.,
583 2015). However, no negative controls were used to exclude substrate-independent
584 signals which could mean that the actual substrate dependent signal was
585 significantly lower. In another study, H₂O₂ production in isolated mitochondria has
586 been measured in the presence of different SA concentrations and different
587 substrates for complex I and complex II (Nie et al., 2015). A very high induction of
588 H₂O₂ production was shown after SA was added, but this study also lacks a negative
589 control without substrate. Therefore, the scale of the measured signals in these
590 reports might need reconsideration as they could be substrate-independent and
591 might be mainly caused by background signals occurring in both assays.

592

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

605 The interplay between SA and H₂O₂ and which of these molecules acts first in plant
606 defence appears to vary depending on the pathway being examined (Vlot et al.,
607 2009). We have previously shown that GSTF8 regulation is H₂O₂-dependent
608 (Gleason et al. (2011), Supplemental Fig. 1 bottom) and that accumulation of H₂O₂
609 follows the SA effect and quantitatively depends on the degree of function of the
610 mitochondrial SDH complex. Earlier studies also showed that SA can enhance H₂O₂
611 production (Shirasu et al., 1997). Recent studies identified GSTF8 as a SA binding
612 protein (Manohar et al., 2014, Tian et al., 2012) but the biological consequences of
613 that interaction and whether it is involved in stress signalling remains unclear. Based

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

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

630

631 **Materials and Methods**

632

633 **Growth of Arabidopsis hydroponic plants**

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

643

644 **GSTF8:luciferase signalling of Arabidopsis seedlings**

645 4 days old seedlings of WT, *dsr1* and *sdhaf2* (in the JC66 background) were grown
646 on MS media plus luciferin1/2 MS medium without vitamins, 1% (w/v) sucrose, pH

647 7.0, 50 μ M luciferin (Biosynth AG)) with without malonate or succinate using 92x16
648 mm petri dishes as described previously (Gleason et al., 2011). After incubation with
649 7 mM SA for 40 min, whole plant bioluminescence was captured over 24 hours using
650 a NightShade imager (Berthold Technologies, Bad Wildbad, Germany) with data
651 calculated in average light units (counts/sec) per seedling using IndiGo (v 2.0.3.0)
652 software (Berthold Technologies).

653

654

655 **Isolation of mitochondria from hydroponic cultures**

656 Mitochondria were isolated from 2 weeks old hydroponically grown Arabidopsis
657 plants using a previously described method from Millar et al. (2001), with slight
658 modifications. Plant material was homogenized in grinding buffer (0.3 M sucrose, 25
659 mM tetrasodium pyrophosphate, 1% (w/v) PVP-40, 2 mM EDTA, 10 mM KH_2PO_4 ,
660 1% (w/v) BSA, 20 mM ascorbic acid, pH 7.5) using mortar and pestle for 2 to 5 min,
661 twice. The homogenate was filtered through four layers of Miracloth and centrifuged
662 at 2500 x g for 5 min, the resulting supernatant was then centrifuged at 14,000 x g
663 for 20 min. The resulting pellet was resuspended in sucrose wash medium (0.3 M
664 sucrose, 0.1% [w/v] BSA, 10 mM TES (N-tris[hydroxymethyl]- methyl-2-
665 aminoethanesulfonic acid], pH 7.5) and carefully layered over 35 ml PVP-40 gradient
666 (30% Percoll, 0 – 4% PVP). The gradient was centrifuged at 40 000 x g for 40 min.
667 The mitochondrial band was collected and washed 3 times in sucrose wash buffer
668 without BSA at 20 000 x g for 20 min.

669

670 **Measurement of SDH activity and kinetic calculations**

671 SDH activity was measured directly at the subunit *SDH1-1* by succinate dependent
672 DCPIP reduction at 600 nm. Isolated Arabidopsis mitochondria (50 μ g) were used in
673 1 ml of reaction medium (50 mM potassium phosphate pH 7.4, 0.1 mM EDTA, 0.1%
674 (w/v) BSA, 10 mM potassium cyanide, 0.12 mM dichlorophenolindophenol (DCPIP)
675 and 1.6 mM phenazine methosulfate (PMS)). To calculate SDH activity, an extinction
676 coefficient of 21 $\text{mM}^{-1} \text{cm}^{-1}$ at 600 nm for DCPIP was used. Brooks Kinetic Software
677 and linear Hanes-Plot calculations were used for kinetic calculations. For
678 measurements targeting the UQ binding site of SDH (SQR activity), 80 μ m
679 Coenzyme Q₁ instead of PMS was used in the reaction medium (Miyadera et al.,
680 2003).

681

682 **Measurement of Complex III activity**

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

690

691 **Measurement of oxygen consumption using an O₂ Clark electrode**

692 Oxygen consumption was measured using an O₂ Clark electrode. Isolated
693 Arabidopsis mitochondria (100 µg) were used and oxygen uptake measured as
694 previously described in Huang et al. (2013) in the presence of either 5 mM succinate,
695 1 mM NADH or 10 mM malate+ glutamate. To investigate the effect of SA on
696 respiration, concentrations from 0.01- 1 mM were added after the substrate.

697

698 **Mitochondrial ROS measurements using DCFDA**

699 DCFDA (2', 7' -dichlorofluorescein diacetate), a cell permeant reagent, which is
700 reacting with ROS within the cell, was used. DCFDA is deacetylated by cellular
701 esterases and forms the fluorescent compound 2', 7' -dichlorofluorescein (DCF)
702 once it is oxidized by ROS. DCF can be detected by fluorescence spectroscopy
703 using excitation/emission spectra of 480/520 nm. Freshly isolated mitochondria (10
704 µg) from hydroponically grown Arabidopsis plants (continuously in the dark), were
705 transferred in 50 µl buffer (0.3 M sucrose, 5 mM KH₂PO₄, 10 mM TES, 10 mM NaCl,
706 2 mM MgSO₄, 0.1 % (w/v) BSA, pH 7.2). DCFDA was diluted to 10 µM a final volume
707 of 50 µl in the same buffer solution together with the individual substrates. Both
708 solutions were transferred and mixed in a 96 well plate to a final volume of 100 µl.
709 Fluorescence was measured over 10 min and the slope was calculated.

710

711 **Figure legends**

712

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

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

720

721

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

723 Concentrations of 0.1 to 10 mM of succinate were used to calculate maximal SDH
724 activity, measured as absorbance change of DCPIP at 600 nm. K_m was calculated
725 using Hanes-Plot and Brook Kinetics Software. (A) Scheme of SDH showing electron
726 transfer from succinate to UQ binding site. (B) Correlation of SDH activity and
727 succinate concentrations of WT, *sdhaf2* and *dsr1*. (C) Maximal enzyme velocity
728 (V_{max}). (D) Calculated K_m of succinate using Brooks kinetic software. (E) enzymatic
729 efficiency (V_{max}/K_m) for *sdhaf2* and *dsr1*. Standard error (SEM) of 6 biological
730 replicates; Two-factor ANOVA comparing SDH Activity between genotypes (B) $p \leq$
731 0.01 (*dsr1* compared to WT and *sdhaf2*), Single-factor ANOVA comparing catalytic
732 efficiency and succinate affinity (D, E) between genotypes. Different letters indicate
733 significant differences ($p \leq 0.05$) between genotypes. n.d. not detected

734

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

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

744

745

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

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

752

753

754 **Figure 5: Low concentrations of SA increase SQR activity.**

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

766

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

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

775

776

777

778 **Supplemental Materials**

779

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

781

782 Supplemental Figure 2: Inhibition of competitive inhibitor malonate in the presence of
783 5 mM succinate.

784

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

787

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

789

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

791

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

794

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

797

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

800

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

803

804

805

806

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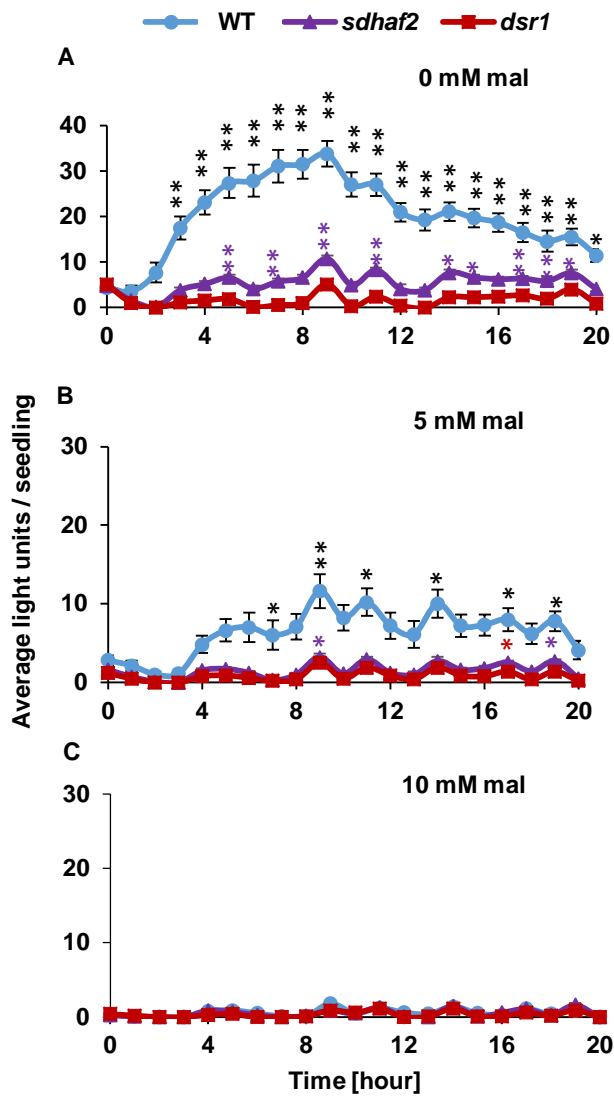
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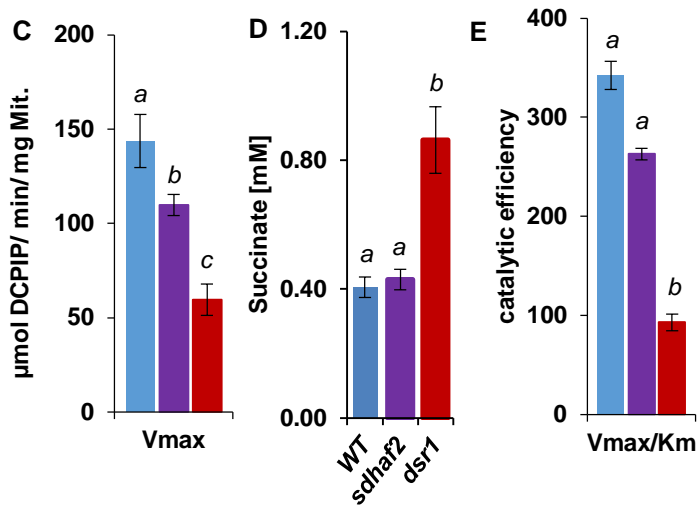
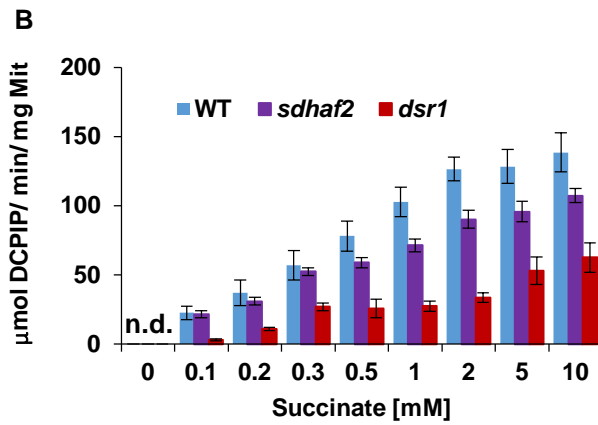
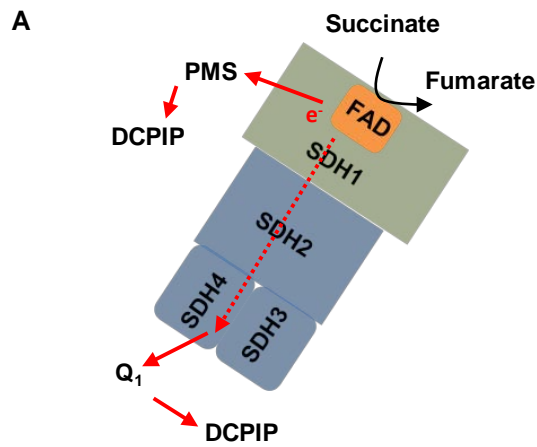
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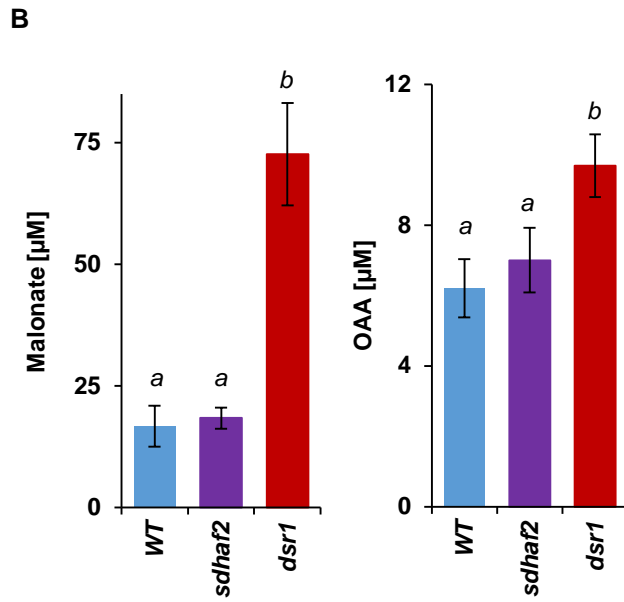
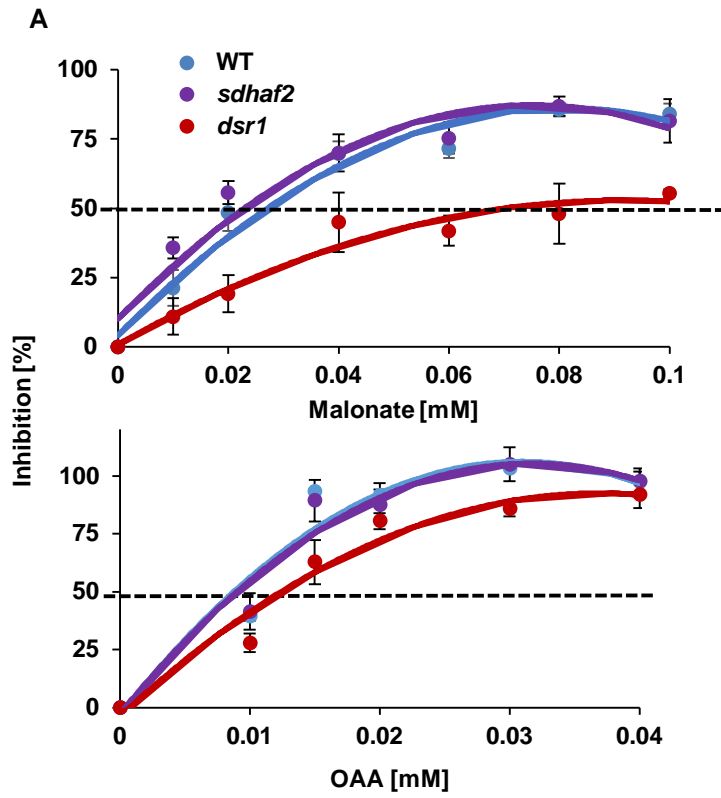
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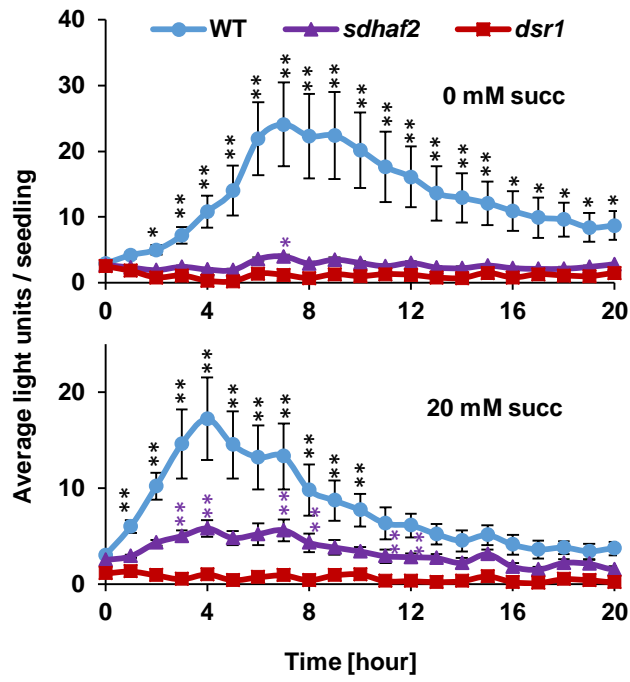
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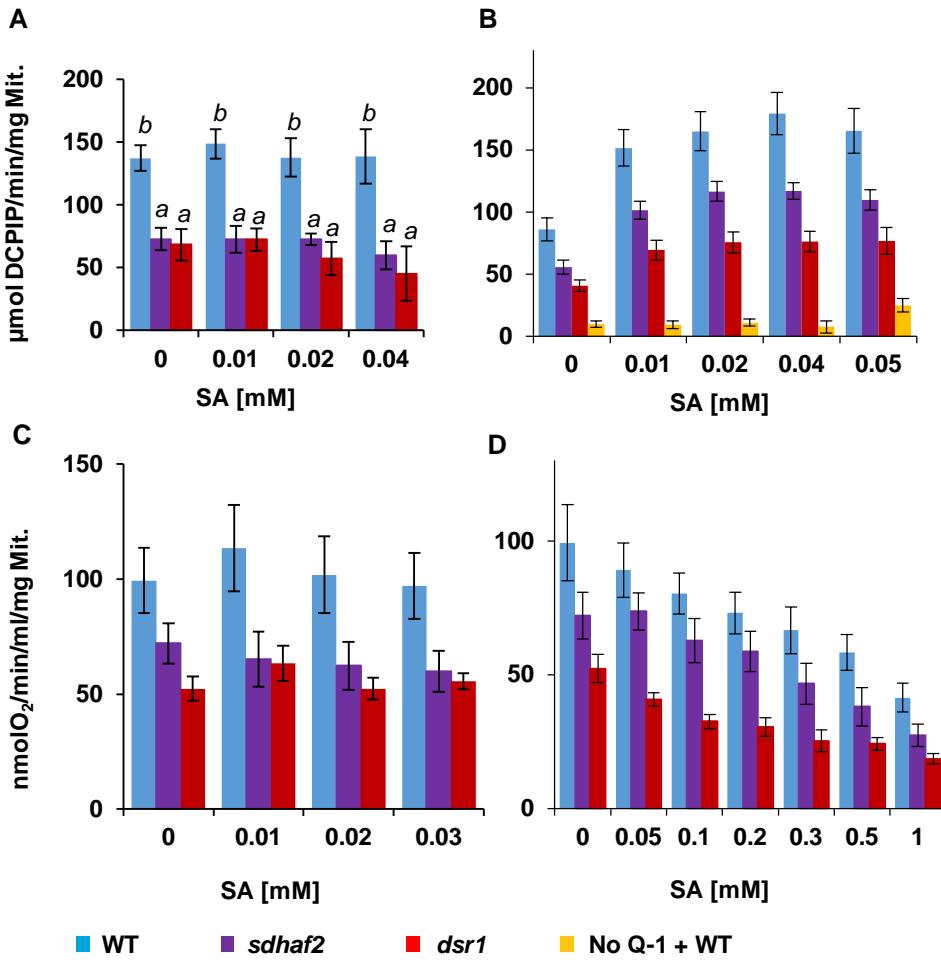
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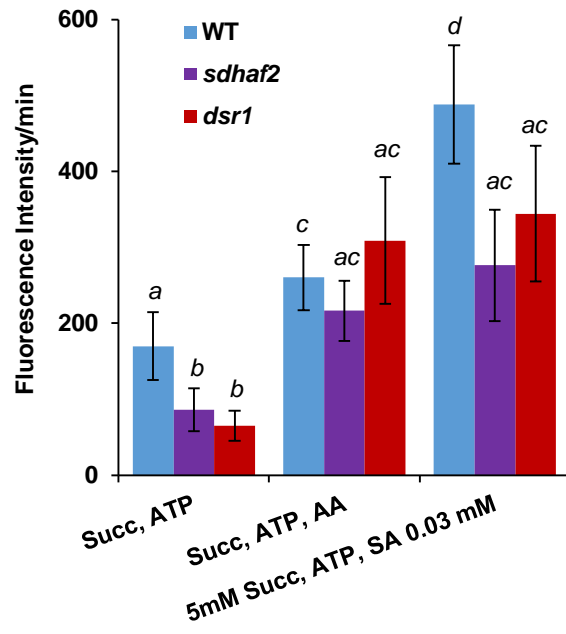












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