Mitochondrial complex II has a key role in mitochondrial-derived reactive oxygen species influence on plant stress gene regulation and defense

Cynthia Gleason[a,1], Shaobai Huang[b,1], Louise F. Thatcher[a,c], Rhonda C. Foley[a], Carol R. Anderson[a,2], Adam J. Carroll[d], A. Harvey Millar[b,3], and Karam B. Singh[a,e,3]

[a]Commonwealth Scientific and Industrial Research Organisation (CSIRO) Plant Industry, Wembley, WA 6913, Australia; [b]Australian Research Council (ARC) Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley, WA 6009, Australia; [c]Queensland Bioscience Precinct, CSIRO Plant Industry, St Lucia, QLD 4067, Australia; [d]ARC Centre of Excellence in Plant Energy Biology, Australian National University, Canberra, ACT 2600, Australia; and [e]University of Western Australia Institute of Agriculture, University of Western Australia, Crawley, WA 6009, Australia

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Mitochondria are both a source of ATP and a site of reactive oxygen species (ROS) production. However, there is little information on the sites of mitochondrial ROS (mROS) production or the biological role of such mROS in plants. We provide genetic proof that mitochondrial complex II (Complex II) of the electron transport chain contributes to localized mROS that regulates plant stress and defense responses. We identify an *Arabidopsis* mutant in the Complex II subunit, SDH1-1, through a screen for mutants lacking *GSTF8* gene expression in response to salicylic acid (SA). *GSTF8* is an early stress-responsive gene whose transcription is induced by biotic and abiotic stresses, and its expression is commonly used as a marker of early stress and defense responses. Transcriptional analysis of this mutant, disrupted in stress responses 1 (*dsr1*), showed that it had altered SA-mediated gene expression for specific downstream stress and defense genes, and it exhibited increased susceptibility to specific fungal and bacterial pathogens. The *dsr1* mutant also showed significantly reduced succinate dehydrogenase activity. Using in vivo fluorescence assays, we demonstrated that root cell ROS production occurred primarily from mitochondria and was lower in the mutant in response to SA. In addition, leaf ROS production was lower in the mutant after avirulent bacterial infection. This mutation, in a conserved region of SDH1-1, is a unique plant mitochondrial mutant that exhibits phenotypes associated with lowered mROS production. It provides critical insights into Complex II function with implications for understanding Complex II's role in mitochondrial diseases across eukaryotes.

plant defense | respiration | *Pseudomonas syringae* | *Rhizoctonia solani*

Plants are barraged by biotic and abiotic stresses that can cause large annual losses to global food production, and, as a result, plants have evolved mechanisms to quickly perceive and respond to these external stresses (1, 2). At the gene-expression level, plant responses to many biotic and abiotic stresses share considerable overlap, particularly during the early stages of the response, and these responses are controlled by overlapping sets of signaling molecules that include plant hormones, calcium, nitric oxide, and reactive oxygen species (ROS) (3–5). The interplay between these signaling molecules and their respective downstream networks is complex and not fully understood.

One group of genes that are induced by combinations of stress signaling pathways are the glutathione *S*-transferases (GSTs). GSTs encode ubiquitous enzymes found in both animals and plants that protect tissues against oxidative damage or from toxins produced during xenobiotic metabolism (6). In plants, the transcriptional activation of diverse GSTs can be triggered by chemicals [salicylic acid (SA)], auxinic herbicides (e.g., dicamba), and ROS (H_2O_2) as well as both biotic (fungal and bacterial elicitors) and abiotic stresses (6). A good example is *GSTF8*, which has been used as a marker for early stress/defense gene induction (7–9). Mutants with altered *GSTF8* expression could provide critical information about the regulation of gene expression such as novel transcription factors or other upstream regulatory components with roles in plant defense and/or stress responses.

To gain insights into conserved aspects of biotic, abiotic, and chemical signaling pathways, we conducted a forward genetic screen to identify mutants with changes in *GSTF8* promoter activity. In this work, we have characterized an *Arabidopsis* mutant that showed loss of inducible *GSTF8* expression in response to stresses and increased susceptibility to fungal and bacterial pathogens. In mitochondrial complex II (Complex II), also known as succinate dehydrogenase (SDH), we mapped the mutation to the catalytic subunit (SDH1-1), providing genetic proof that the mitochondrial respiratory electron transport chain (10, 11) contributes to the propagation of plant stress and defense responses.

Results

Identification of an *Arabidopsis* Mutant with Altered Stress Gene Responsiveness. *GSTF8* promoter activity can be monitored with an *Arabidopsis thaliana* (Columbia-0) transgenic line (JC66) in which 791 bp of the *GSTF8* promoter has been fused to a luciferase (LUC) reporter (12, 13). Approximately 100,000 M2 seedlings from ethyl methanesulfonate-mutagenized seeds of JC66 were screened for altered SA induction of the *GSTF8* promoter by monitoring whole-plant luminescence 4 h after SA treatment. Strong promoter activity was observed primarily in the roots of WT (JC66) plants in response to SA (Fig. 1*A*). We focused our attention to a loss-of-function mutant, which showed almost no SA-induced *GSTF8* promoter activity, and called this mutant disrupted in stress responses 1 (*dsr1*).

To further characterize the *dsr1* mutation, the *GSTF8*::LUC response to SA was monitored over a 14-h time course (Fig. 1*A*). In WT, there was *GSTF8* promoter activity after SA treatment, peaking at 8–12 h after treatment. In contrast, the *dsr1* mutant had significantly less promoter activity induced by SA (Fig. 1*A*).

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[1]C.G. and S.H. contributed equally to this work.

[2]Deceased January 24, 2009.

[3]To whom correspondence may be addressed. E-mail: harvey.millar@uwa.edu.au, or karam.singh@csiro.au.

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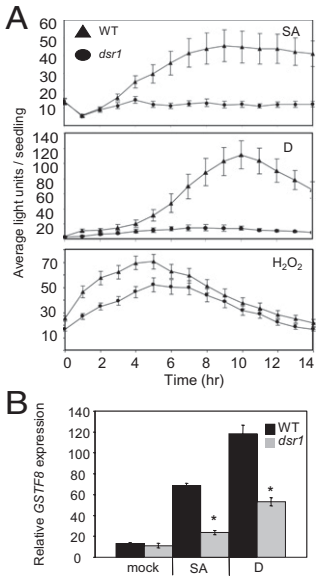

Fig. 1. *dsr1* shows altered *GSTF8* induction in response to certain stresses. (*A*) Average of the total bioluminescence generated by each seedling (n = 20) per hour after treatment with SA, dicamba (D), or H_2O_2. (*B*) Relative expression of *GSTF8* in WT and *dsr1* plants at 10 h after treatment with water (mock), SA, or dicamba (D). Gene-expression experiments using three biological and two technical repeats were repeated twice with similar results. [Error bars: standard error (SEM); Student's t test, $*P < 0.05$.]

Furthermore, the SA analog 2,6-dichloroisonicotinic acid and the SA precursor benzoic acid also induced *GSTF8* promoter activity in WT plants to much higher levels than in the *dsr1* mutant (Fig. S1). Treatment of WT seedlings with the auxin-like herbicide dicamba induced *GSTF8* promoter activity, but this induction was largely absent in *dsr1* seedlings (Fig. 1*A*). A decrease in both SA and dicamba-induced *GSTF8* transcriptional levels in *dsr1* compared with WT confirmed the LUC results (Fig. 1*B*). In contrast, the induction of the *GSTF8* promoter activity by H_2O_2 seen in WT seedlings also occurred in *dsr1* (Fig. 1*A*), indicating that the *dsr1* mutation affected *GSTF8* promoter responses to some but not all inducers. The *dsr1* plants did not display any abnormal growth or developmental phenotypes, with *dsr1* plants looking similar to WT (Fig. S2). With regard to abiotic stress responses, the *dsr1* plants showed no difference in root-inhibition assays in response to NaCl and dicamba, but *dsr1* did show some resistance to mannitol-induced osmotic stress (Fig. S2).

To better understand the loss of SA-induced *GSTF8* expression in *dsr1*, we performed ATH1 microarray analysis on WT and *dsr1* at 10 h after treatment with either water (mock) or 1 mM SA. We observed maximal SA-induced *GSTF8* promoter activity in WT plants at 10 h (Fig. 1*A*). After the mock (water) treatment, two genes were up-regulated ≥2-fold in *dsr1* compared with WT. They encoded a UDP-glucuronosyl/UDP-glucosyl transferase family protein and a nuclear transport factor 2 family protein/RNA recognition motif-containing protein. In the mock treatment, 18 genes in *dsr1* compared with WT were repressed. The largest group (eight genes) was involved in plant development, and four encoded stress-responsive genes (Dataset S1, Table S1*A*).

Global gene-expression analysis revealed that there was >100-fold more genes differentially expressed between *dsr1* and WT upon SA treatment than in the mock treatment (Fig. S3). The largest class of up-regulated genes in SA-treated *dsr1* were abiotic stress related and included a large proportion of genes encoding small heat shock proteins (Dataset S1, Table S1*B*). Overexpression of small heat shock proteins has been directly linked to abiotic stress tolerance phenotypes in *Arabidopsis* (14), which might explain the mannitol-induced osmotic stress tolerance of *dsr1* (Fig. S2).

Interestingly, there were a greater number of genes specifically repressed than induced in SA-induced *dsr1* plants compared with WT. When looking at repressed genes in SA-treated *dsr1* plants, a large number of genes were involved in plant development and biotic stress responses. Peroxidases, glutaredoxins, and trypsin and protease inhibitor family genes comprised some of the biotic stress-responsive genes that were significantly repressed in the mutant (Dataset S1, Table S1*B*).

In an alternative means of analysis, we considered the 100 most SA-induced genes in WT whose fold induction ranged from 6.7 to 163 and looked at the transcriptional response of these genes in *dsr1* (Dataset S1, Table S1*C*). Although many responded similarly, a subset of 18 exhibited significantly lower or no induction in *dsr1* (Fig. S3 and Dataset S1, Table S1*C*). This set of genes contained largely known SA-responsive genes from published reports, and more than half are normally induced in response to exposure of *Arabidopsis* to bacterial, fungal, or viral pathogens based on analysis of Genevestigator datasets (Dataset S1, Table S1*D*).

***dsr1* Is More Susceptible to Fungal and Virulent Bacterial Pathogens.** Because the *dsr1* plants have altered SA-regulated gene expression and SA is an important plant defense signaling molecule, these plants may have altered defense responses to pathogens. Therefore, we challenged *dsr1* with different plant pathogens. The necrotrophic root fungus *Rhizoctonia solani*, which causes bare patches and seedling dampening off, is divided into anastomosis groups (AG) based on the ability of the fungal strains to fuse (15). Previously, we found that WT (Columbia-0) plants are resistant to strain AG8 and susceptible to strain AG2 (16). Interestingly, although *GSTF8* was induced after inoculation with the nonpathogenic strain AG8, its induction was absent after inoculation with the pathogenic strain AG2, suggesting that *GSTF8* promoter activity was linked to the resistance response (16). We found that the *GSTF8* promoter was less responsive to AG8 in *dsr1* compared with WT (Fig. 2*A*) and that the average survival of plants inoculated with this root pathogen was significantly lower for *dsr1* compared with WT (Fig. 2*B*). To determine whether *dsr1* has a role in resistance to a foliar pathogen, we studied the disease progression of the virulent bacterium *Pseudomonas syringae* pv.

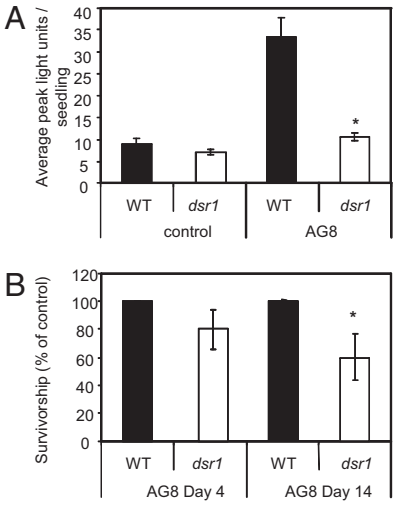

Fig. 2. *dsr1* shows enhanced susceptibility to a fungal root pathogen. (*A*) Average peak induction of the *GSTF8*::LUC promoter activity in WT and *dsr1* in response to *R. solani* AG8. (*B*) Percentage of plant survivorship of WT and *dsr1* seedlings at 4 and 14 d after transfer to vermiculite infested with *R. solani* AG8 (error bars: SEM, Student's t test, $*P < 0.05$).

PLANT BIOLOGY

tomato DC3000 (*Pst* DC3000). At 3 d after leaf inoculation, the *dsr1* plants had higher bacterial growth compared with WT, with bacterial populations >10-fold higher at 3 d postinoculation (Fig. S4*A*). To determine whether *dsr1* was also susceptible to a foliar fungal pathogen, we measured lesion sizes on leaves inoculated with *Alternaria brassicicola*. Although WT leaves developed small necrotic lesions no larger than the initial inoculation droplet, *dsr1* plants developed significantly larger lesions (Fig. S4 *B* and *C*).

***dsr1* Contains a Mutation in a Subunit of SDH.** The *dsr1* mutation was fine-mapped to the distal end of chromosome 5 to a region of ~150 kb. Several genes in this area of the chromosome were sequenced. Gene At5g66760 had a single base-pair mutation (G to A) converting amino acid 581 from alanine to threonine. At5g66760 encodes the flavoprotein subunit of SDH (SDH1-1), one of four subunits comprising Complex II of the electron transport chain in mitochondria. The site of the *dsr1* mutation (Ala-581), and the surrounding sequence, has been widely conserved and is part of the SDH1-1 flavoprotein C-terminal fold involved in substrate binding (Fig. S5) (17).

We applied malonate (a specific inhibitor of SDH) to WT (JC66) plants and found that malonate pretreatment inhibited dicamba- and SA-induced *GSTF8* promoter activity (Fig. 3*A*), providing independent evidence that dicamba- and SA-driven *GSTF8* expression relies, at least in part, on SDH function. To further confirm that *dsr1* was due to the mutation in *SDH1-1*, we generated nine independent T2 lines in which the *SDH1-1* cDNA was expressed from the 35S promoter in the *dsr1* mutant background. Treatment with dicamba significantly increased *GSTF8* promoter activity in eight of nine of these complemented lines compared with *dsr1* but did not fully restore levels to WT (Fig. S6*A*). We concentrated work on two independent T3 lines, each having much stronger relative expression of SDH1-1 than WT did (Fig. S6*B*) and significant levels of restoration of GSTF8 promoter activity (Fig. 3*B*).

Previously, it was reported that heterozygous mutants for SDH1-1 by transfer DNA insertion showed altered gametophyte development and pollen abortion and that homozygous mutants for disruption of SDH1-1 could not be obtained because of the *sdh1-1* allele not being transmitted through the male gametophyte and being only partially transmitted through the female gametophyte (18). We did not observe obvious phenotypic differences between *dsr1* and WT plants (Fig. S2) and saw no changes in pollen viability (Fig. S6*C*). There are two SDH1 genes in *Arabidopsis*, *SDH1-1* (At5g66760) and *SDH1-2* (At2g18450), but only SDH1-1 has been found in the SDH protein complex in mitochondria to date (19). The relative gene expression for *SDH1-1* was much higher than that of *SDH1-2*, and there was no compensatory expression of *SDH1-2* in *dsr1* plants, nor was there any induction of either gene by SA or dicamba (Fig. S6*D*). Also, previous studies showed that knockout of SDH1-2 had no effect on growth or development in *Arabidopsis* (18).

***dsr1* Plants Have Defective SDH, Affecting Mitochondrial Electron Transport Chain Activity.** *dsr1* plants have a mutation in *SDH1-1* that may result in aberrant plant mitochondrial phenotypes. There was no significant difference in root respiration rates between WT and *dsr1* (Fig. 3*C*). When SA was applied, however, an inhibition of root respiration occurred for *dsr1* but not for WT (Fig. 3*C*). To specifically analyze SDH activity, mitochondria were isolated, and the succinate-dependent reduction of dichlorophenolindophenol (DCPIP) in *dsr1* was only 20% of that from WT, whereas the two complemented lines had a partial restoration in SDH activity (Fig. 3*D*), suggesting that the presence of the mutant SDH1 protein and the multisubunit structure of Complex II prevents full complementation of the *dsr1* phenotypes in the complemented lines.

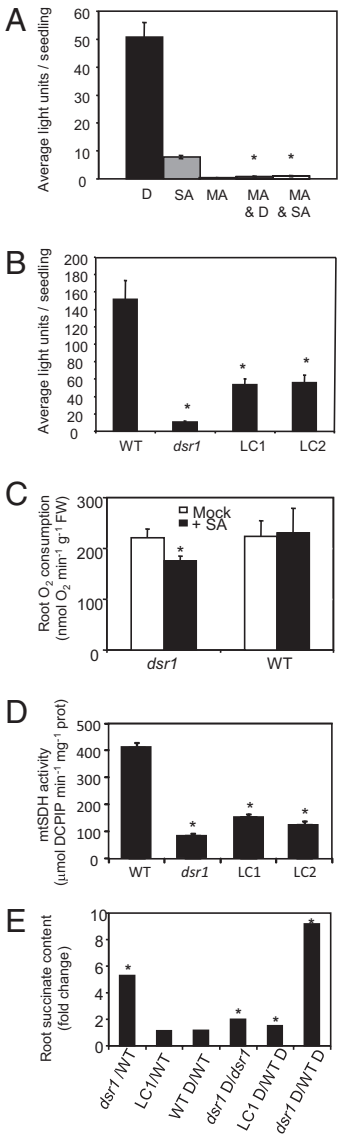

Fig. 3. Characterization of the *dsr1* mutation showing decreased respiration and SDH function. (*A*) Inhibition of *GSTF8* promoter activity induced by dicamba (D) and SA after pretreatment of seedlings with 10 mM malonate (MA). The average bioluminescence (light units) was measured per seedling after a 2-h pretreatment with H_2O or malonate, followed by a 4-h second treatment with dicamba, SA, or malonate. MA & D, malonate followed by dicamba; MA & SA, malonate followed by SA. (*B*) Measurement of bioluminescence from seedlings from WT, *dsr1*, and At5g66760 cDNA in two *dsr1* complemented backgrounds (LC1 and LC2) at 4 h after treatment with 7 mM dicamba. ($n = 12$; error bars: SEM; repeated three times.) (*C*) Root respiration rate and effect of 1 mM SA compared with mock (water) treatment. (*D*) SDH activity in isolated mitochondria in the presence of 0.5 mM ATP. (*E*) Succinate content of roots and in response to dicamba (D). (*$P < 0.05$.)

To determine whether this alteration in one component of the mitochondrial electron transport chain could alter respiratory rate, we further analyzed mitochondrial respiratory activity in *dsr1* and showed that the succinate-dependent O_2 consumption rate of intact isolated mitochondria was 40% that of WT (and was partially restored in the complemented lines; Dataset S1, Table S1*E*). However, there was no significant difference in the NADH-dependent O_2 consumption rate of isolated mitochondria (Dataset S1, Table S1*E*), suggesting that a changed capacity of the rest of the electron transport chain was not the cause for the *dsr1* mitochondrial respiratory phenotype. Direct analysis of

SDH showed that there was no obvious change in the size or abundance of the native SDH complex or the incorporation of FAD into SDH1 (Fig. S7*A*), although there appeared to be lower in-gel SDH activity in *dsr1* (Fig. S7*B*). The abundance of SDH1-1 and other SDH complex subunits did not significantly change between WT and *dsr1* mitochondrial samples based on comparative analysis of blue native (BN)-SDS/PAGE separation of electron transport chain complexes (Fig. S7*B*). A fully quantitative analysis of the proteome of WT, *dsr1*, and complemented line (LC1) mitochondria using fluorescence labeling with Cy dyes showed no apparent compensatory changes and, notably, that the overall levels of SDH1-1 protein (native and mutant SDH1-1) were equal in WT, *dsr1*, and LC1 (Fig. S7*C*). We have recently reported the direct ATP activation of SDH activity in *Arabidopsis* and the ATP-binding capacity of the *Arabidopsis* SDH1-1 protein (19). The activation of O_2 consumption rates and SDH enzymatic activities by ATP were not observed in *dsr1*, indicating that the *dsr1* point mutation has abolished this activation of SDH (Dataset S1, Table S1*E*).

Analysis of whole-root methanol-soluble extracts by GC-MS profiling identified and quantified 51 known metabolites (Dataset S1, Table S1*F*). Succinate was approximately fivefold and ninefold higher in abundance in *dsr1* than in WT mock and dicamba-treated extracts, respectively (Fig. 3*E*) and was, by far, the most strongly affected metabolite in *dsr1* (Dataset S1, Table S1*F*). In *dsr1*, dicamba treatment itself also raised succinate levels by twofold at 0.5 h posttreatment (Fig. 3*E*), preceding the peak in promoter activity (Fig. 1*A*). Succinate content returned close to WT levels in the complemented line (LC1). In combination, these data indicate a metabolic block at the level of succinate utilization in vivo in *dsr1* and that dicamba treatment likely exacerbates this effect, indicating a dicamba-induced metabolic flux enhancing succinate generation in *dsr1*. More minor differences in other intermediates in tricarboxylic acid metabolism, such as malate, 2-oxoglutarate, and citrate were also recorded in *dsr1* (Dataset S1, Table S1*F*), further suggesting a rate limitation of SDH for tricarboxylic acid cycle metabolism in the mutant.

***dsr1* Plants Have Diminished Mitochondrial H_2O_2 (mH_2O_2) Production.** Because H_2O_2 can restore *GSTF8* promoter activity in *dsr1* (Fig. 1*A*), we hypothesized that *dsr1* might have less production of mH_2O_2 than WT. We measured the succinate-dependent H_2O_2 production rate by isolated mitochondria using the Amplex Red and peroxidase-coupled method (20). ATP stimulated succinate-dependent H_2O_2 production in WT but not in *dsr1* (Dataset S1, Table S1*E*). WT had a threefold higher rate of succinate-dependent H_2O_2 production than that observed in *dsr1* in the presence of ATP (Fig. 4*A*). H_2O_2 production in *dsr1* mitochondria was reduced significantly compared with WT under normal growth conditions and after stress treatments (Fig. 4*A*).

Plants treated with SA or dicamba are known to accumulate ROS. Our results suggested that the block in *GSTF8* promoter induction in *dsr1* after SA or dicamba treatment may be because of a specific loss of H_2O_2 production from mitochondria. This finding is further supported by evidence that WT (JC66) seedlings simultaneously treated with catalase (for removal of H_2O_2) and either dicamba or SA had a dramatic decrease in promoter activity compared with the application of dicamba or SA alone (Fig. 4*B*).

To provide further support for these findings, we visualized the subcellular location of H_2O_2 production in *Arabidopsis* roots by using dichlorofluorescein diacetate (DCFDA) fluorescence in intact protoplasts of WT and *dsr1*. We counterstained with MitoTracker Red to identify the location of mitochondria (Fig. S8*A*). This staining showed that the majority of in vivo H_2O_2 production is occurring from mitochondria in *Arabidopsis* root protoplasts. We then incubated protoplasts with the chemical treatments of SA or dicamba for 30 min and recorded differences in DCF fluorescence over the following 10 min. In WT but not

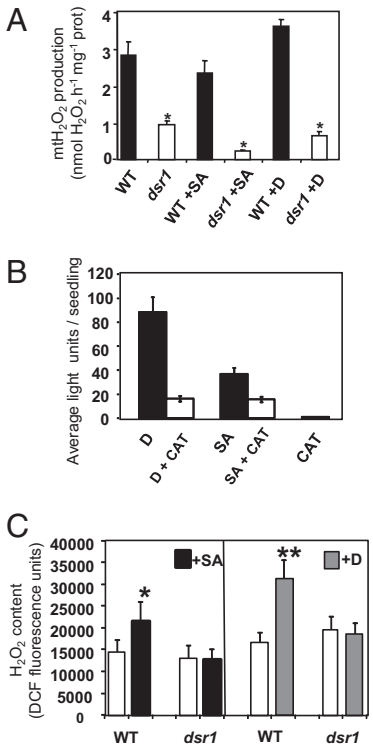

Fig. 4. H_2O_2 production that is required for GSTF8 promoter activity is less in *dsr1*. (*A*) H_2O_2 production from isolated mitochondria incubated with succinate and 0.5 mM ATP and treated with 1 mM SA or 1 mM dicamba (D) as indicated. (*$P < 0.05$.) (*B*) Average bioluminescence in WT seedlings after treatment with dicamba (D), dicamba and catalase (D+CAT), SA alone, SA and catalase (SA+CAT), or catalase alone (CAT). (Error bars: SEM; *$P < 0.05$.) (*C*) Root protoplast average H_2O_2 production in WT and *dsr1* under control conditions and after 30-min chemical treatment with 100 μM SA or 100 μM dicamba (D). (Error bars: SEM; *$P < 0.05$, **$P < 0.005$, Student's *t* test; $n = 26–76$.)

dsr1, these treatments increased H_2O_2 production (Fig. 4*C*), and this ROS originated from mitochondria, as seen in individual root protoplast images (Fig. S8*A*).

Given the difference in the sensitivity of *dsr1* to foliar infection with *P. syringae* (Fig. 3*C*), we were interested in examining whether a biotic stress treatment of *dsr1* resulted in altered H_2O_2 production in foliar tissue. Inoculation of WT plants with avirulent *Pst* initiates a hypersensitive response preceded by an oxidative burst in leaves. To determine whether the *dsr1* mutation alters ROS production during this oxidative burst, we measured H_2O_2 content by 3,3′-diaminobenzidine (DAB) staining in leaves injected with the avirulent bacterial strain *Pst* DC3000 (*avrRpt2*) 1 d after infiltration, an early stage of infection. Quantification of DAB precipitate showed more DAB in WT-infected leaves than in *dsr1*, indicating more H_2O_2 production in WT than in *dsr1* (Fig. S8 *B* and *C*). No staining was shown in either WT or *dsr1* after injection with $MgCl_2$. Despite this decrease in H_2O_2 levels in *dsr1*, bacterial growth measurements did not show significant differences between *dsr1* and WT over 3 d (Fig. S8*D*), and the *dsr1* plants developed disease symptoms similar to WT.

Discussion

***dsr1* Plants Have a Unique Mutation in *SDH1-1*.** The discovery of a point mutation that affects SDH1-1 activity without adversely affecting plant viability illustrates the power of the forward genetic screen using an early stress-responsive promoter::reporter gene system and has given us the capacity to uncover a unique role of ROS production from Complex II in intact plants. *dsr1* plants have a single amino acid change in the conserved C ter-

minus of the protein (A581T) that has the potential to be a more subtle change to the SDH1-1 protein than a RNAi knockdown or transfer DNA insertional mutant. Mitochondria from *dsr1* retained 20% of SDH activity and 40% of WT succinate-dependent respiratory rate (Fig. 3 and Dataset S1, Table S1*E*), and the protein content of Complex II was unchanged (Fig. S7). Although the complete knockout of SDH1-1 is lethal, work on *SDH1-1*/*sdh1-1* heterozygous mutant plants and SDH1-1 RNAi knockdown lines showed defects in gametophyte development (18). However, the *dsr1* plants did not exhibit altered pollen development (Fig. S6*C*) or obvious developmental defects.

Our complementation of the mutant with WT SDH1-1 helped confirm that the *dsr1* point mutation was causing the defects in mH_2O_2 production and *GSTF8* promoter activity. However, there was only a partial complementation of the mutant phenotype because *GSTF8* promoter activity and mitochondrial respiration were not fully restored to WT levels, despite being significantly above *dsr1* levels. We hypothesize that, because Complex II is a multisubunit complex, the overexpressed WT SDH1-1 cannot fully displace *sdh1-1* in the complex and/or possibly titrates out interacting partners, affecting Complex II formation, stability, and/or activity. The fact that the overall levels of SDH1-1 protein is similar in the WT, *dsr1*, and the complemented line (Fig. S7), despite high levels of SDH1-1 transcript in the complemented lines (Fig. S6), suggests that substantial posttranscriptional control is occurring, which could affect the ability to fully complement the mutant phenotype. Alternatively, this lack of full complementation could suggest that a tightly linked additional mutation is affecting the *dsr1* mutant. However, we consider that our evidence is very strongly in favor of SDH1 as the functional site of the effects we show given the fine mapping we have performed on the mutant, qualitative complementation, and the functional evidence of altered SDH function and phenocopying with SDH inhibitors.

According to the 3D structure of SDH1 from mammals, this A581T mutation is in the substrate-binding pocket for succinate and the known SDH inhibitors oxaloacetate and malonate (Fig. S5). Reports of mutations in SDH1 protein in eukaryotes are rare and usually are associated with severe metabolic disorders but not tumorigenesis (21). A notable one is the homozygous mutation in humans, R554Y, which leads to ∼50% decrease in SDH activity, a loss of activation of SDH by ATP, and enhanced oxaloacetate inhibition in isolated mitochondria as well as results in a Leigh-like syndrome in patients (22). A SHD1 R589W mutation has been reported in a case of catecholamine-secreting abdominal paraganglioma, leading to lowered SDH activity and a pseudohypoxia response (23). There are no previous reports of a paraganglioma case associated with SDH1, in contrast to many reported mutations in SDH2, -3, and -4 that are associated with hereditary paraganglioma/pheochromocytoma and elevated mH_2O_2 production (21, 24). Our in-depth analysis of *dsr1* could help in explaining this anomaly in disease outcomes by providing additional evidence that specific modifications in this substrate-binding site appear to lower both SDH activity and ROS production, rather than enhance it. In *Arabidopsis*, succinate-dependent mH_2O_2 production was also increased by ATP in WT but not *dsr1*, again implicating the substrate-binding pocket in modulating H_2O_2 production by SDH.

A number of viable respiratory mutants in plants have been reported, most notably cytoplasmic male sterile mutants (25), but few have been extensively analyzed in terms of mitochondrial function and ROS production phenotypes. Exceptions to this rule are mutants leading to the loss of Complex I that can be compensated for in plants because of external and internal rotenone-insensitive dehydrogenases (26). Such mutants have altered abiotic stress phenotypes and either altered cellular redox networks (27) or increased ROS levels (28). This leaves *dsr1* as the only plant electron transport chain mutant to date with phenotypes associated with lowered mitochondrial ROS (mROS) production, thus providing valuable insights into the role of mROS in plants.

mH_2O_2 Contributes to Plant Defense to Fungal and Bacterial Pathogens. Plant mitochondria have been previously implicated in generating ROS during pathogen attack and hypersensitive responses. For example, treatments of mitochondria with harpin or avirulent *Pst* were reported to cause the production of mROS, change the membrane potential, and inhibit ATP synthesis (29, 30). However, the typically debilitating effect of respiratory chain knockout mutants has made it difficult to define the mechanism by which mitochondrial activity impacts pathogen defenses.

The increased susceptibility of *dsr1* to a fungal pathogen of the root, *R. solani* AG8, and of the leaf, *A. brassicicola*, and to a virulent bacterial foliar pathogen, *Pst* DC3000, directly illustrates the importance of mitochondria function in pathogen defenses in both root and leaf. mROS may contribute to plant defense by either directly acting against the pathogen or acting as a signaling molecule in plant defenses. For a fungal pathogen like *R. solani* AG8, which is unable to penetrate the roots of WT plants (16), mROS could contribute to the oxidative cross-linking of the cell wall to help prevent fungal penetration (31, 32). Moreover, some evidence suggests that signaling may be the primary role of ROS in *R. solani* resistance because resistant transgenic cotton seedlings exhibited a rapid induction of ROS and downstream defense-related genes (33). Although *dsr1* had diminished mH_2O_2 production, it was still resistant to the avirulent bacteria *Pst* DC3000 (*avrRpt2*). The resistance may be because of other H_2O_2 production centers in the plant that were fully functional, thereby allowing H_2O_2 levels to reach a threshold at which pathogen-induced resistance pathways are triggered. Alternatively, plant resistance to this avirulent pathogen may be independent of ROS production. Previous studies have shown that *Arabidopsis* mutants lacking apoplastic ROS production from NADPH oxidases were still resistant to avirulent *P. syringae*, indicating that resistance was independent of NADPH oxidase-generated ROS (34). Our results are consistent with this previous work and show that resistance to *Pst* DC3000 (*avrRpt2*) is also independent of the alteration of mH_2O_2 in *dsr1*.

Complex II Is a Source of mH_2O_2 That Regulates Downstream Defense and Stress Gene Expression. There are multiple sources of ROS in the plant cell and potentially others that remain to be identified. NADPH oxidases are proposed to be the primary source of ROS in the apoplast upon pathogen infection (35). The chloroplasts, peroxisomes, mitochondria, and nuclei all produce ROS, and the multiple sources of ROS suggest that the spatial compartmentalization of ROS pools may play a critical role in regulating specific downstream responses (5, 36–38). In the green organs of plants, the ROS are generated mainly by chloroplasts and peroxisomes, but in nonphotosynthetic plant cells, the mitochondrial electron transport chain is a key site of the production of ROS, with 1–3% of the total oxygen consumption leading to the generation of ROS rather than H_2O (26).

However, even within plant mitochondria there are contradictory reports about the sites of ROS production (26). The ROS production by mitochondria is generally associated with a high level of reduction of mobile electron carriers, or cytochromes/Fe-S centers/flavins, within respiratory chain protein complexes. Electron transport through mitochondrial Complexes I and III are often considered the main sites of mROS production from the electron transport chain (39). However, it has also been reported that succinate-dependent mH_2O_2 production is faster than pyruvate/malate-dependent mH_2O_2 production in plants, indicating that Complex II has a larger role than Complex I (40). Additionally, the ubiquinone pool itself has been highlighted as a site of ROS production in plant mitochondria (41).

This complexity of ROS-generation pathways is further complicated by the interplay between H_2O_2 and SA as well as contradictory evidence as to whether SA acts upstream of H_2O_2 or vice versa (42). Although there are data that H_2O_2 induces SA

accumulation (43), SA also enhances H_2O_2 production (44), and H_2O_2 production can occur in the apoplast, mitochondria, and/or chloroplast (42, 45). Here, we have shown that *GSTF8* requires mH_2O_2 for its gene expression and that SA can be translated into an SDH1-dependent ROS signal by the mitochondria for downstream stress signaling responses and an enhanced plant defense capacity. The unique nature of this SDH1 mutant broadens our understanding of SDH mechanisms and may provide insights into SDH-mediated mitochondrial perturbations across eukaryotes.

Materials and Methods

Approximately 100,000 M2 seedlings from ethyl methanesulfonate-mutagenized seeds of JC66 (containing a *GSTF8*::LUC promoter construct) were screened by bioluminescence to identify *dsr1* as a loss-of-function mutant (nil or low LUC activity after hormone treatment). Gene expression in WT and *dsr1* was analyzed by quantitative RT-PCR, and microarrays were performed per a standard Affymetrix protocol. *R. solani* AG8 was grown in culture, and seedlings sown into vermiculite were inoculated by watering. *A. brassicicola* spore suspension was drop-inoculated onto leaves, and *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*) were syringe-infiltrated into leaves.

Mitochondria were isolated from WT and *dsr1* by using Percoll density gradients. Respiration, enzyme assays, and H_2O_2 measurements were undertaken by using Clark-type O_2 electrodes, spectrophotometric and spectrofluorometric analysis of DCPIP reduction, Amplex Red oxidation, and H_2-DCFDA oxidation, respectively. GC-MS profiles of metabolites were analyzed with MetabolomeExpress software (version 1.0; http://www.metabolome-express.org).

Further detailed methods are available in *SI Experimental Procedures*.

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