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

Cynthia Gleason¹,1, Shaobai Huang¹,1, Louise F. Thatchera,c, Rhonda C. Foleya, Carol R. Andersona,2, Adam J. Carrollb, A. Harvey Millarb,3, and Karam B. Singha,e,3

¹Commonwealth Scientific and Industrial Research Organisation (CSIRO) Plant Industry, Wembley, WA 6913, Australia; ²Australian Research Council (ARC) Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley, WA 6009, Australia; ³Queensland Bioscience Precinct, CSIRO Plant Industry, St Lucia, QLD 4067, Australia; ⁴ARC Centre of Excellence in Plant Energy Biology, Australian National University, Canberra, ACT 2600, Australia; and ⁵University of Western Australia Institute of Agriculture, University of Western Australia, Crawley, WA 6009, Australia

Edited by Bob B. Buchanan, University of California, Berkeley, CA, and approved May 20, 2011 (received for review October 27, 2010)

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 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.

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. 1A). 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. 1A). 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. 1A).


The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE22942).

¹C.G. and S.H. contributed equally to this work.
²Deceased January 24, 2009.
³To whom correspondence may be addressed. E-mail: harvey.millar@uwa.edu.au or karam.singh@csiro.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016060108/-/DCSupplemental.


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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. S4). A decrease in both SA and dicamba-induced GSTF8 transcriptional levels in dsr1 compared with WT confirmed the LUC results (Fig. S1B). In contrast, the induction of the GSTF8 promoter activity by H2O2 seen in WT seedlings also occurred in dsr1 (Fig. S4), 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. S4). 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 S1A).

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 S1B). 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 S1B).

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 S1C). Although many responded similarly, a subset of 18 exhibited significantly lower or no induction in dsr1 (Fig. S3 and Dataset S1, Table S1C). 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 S1D).


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. 2A) and that the average survival of plants inoculated with this root pathogen was significantly lower for dsr1 compared with WT (Fig. 2B). 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.

![Figure 1](image1.png)

**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 H2O2. (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.]

![Figure 2](image2.png)

**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).
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. S4A). 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-driven GSTF8 promoter activity (Fig. 3A), 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. S6A). We concentrated work on two independent T3 lines, each having much stronger relative expression of SDH1-1 than WT did (Fig. S6B) and significant levels of restoration of GSTF8 promoter activity (Fig. 3B).

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 (18). We did not observe obvious phenotypic differences between dsr1 and WT plants (Fig. S2) and saw no changes in pollen viability (Fig. S6C). There are two SDH1 genes in Arabidopsis, SDH1-1 (At5g66760) and SDH1-2 (At2g18450), but only SDH1-1 has been found in the SDH1 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. S6D). 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. 3C). When SA was applied, however, an inhibition of root respiration occurred for dsr1 but not for WT (Fig. 3C). To specifically analyze SDH activity, mitochondria were isolated, and the succinate-dependent reduction of dichloroindolinoephoplenol (DCPIP) in dsr1 was only 20% of that from WT, whereas the two complemented lines had a partial restoration in SDH activity (Fig. 3D), 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.

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 O₂ consumption rate of intact isolated mitochondria was 40% that of WT (and was partially restored in the complemented lines; Dataset S1, Table S1E). However, there was no significant difference in the NADH-dependent O₂ consumption rate of isolated mitochondria (Dataset S1, Table S1E), 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

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 H₂O 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.)
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. S7A), although there appeared to be lower in-gel SDH activity in dsr1 (Fig. S7B). 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. S7B). 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. S7C). 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 O₂ 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 S1E).

Analysis of whole-root methanol-soluble extracts by GC-MS profiling identified and quantified 51 known metabolites (Dataset S1, Table S1F). Succinate was approximately fivefold and ninefold higher in abundance in dsr1 than in WT mock and dicamba-treated extracts, respectively (Fig. 3E) and was, by far, the most strongly affected metabolite in dsr1 (Dataset S1, Table S1F). In dsr1, dicamba treatment itself also raised succinate levels by twofold at 0.5 h posttreatment (Fig. 3E), preceding the peak in promoter activity (Fig. 1A). 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 S1F), further suggesting a rate limitation of SDH for tricarboxylic acid cycle metabolism in the mutant.

**dsr1 Plants Have Diminished Mitochondrial H₂O₂ (mH₂O₂) Production.** Because H₂O₂ can restore GSTF8 promoter activity in dsr1 (Fig. 1A), we hypothesized that dsr1 might have less production of mH₂O₂ than WT. We measured the succinate-dependent H₂O₂ production rate by isolated mitochondria using the Amplex Red and peroxidase-coupled method (20). ATP stimulated succinate-dependent H₂O₂ production in WT but not in dsr1 (Dataset S1, Table S1E). WT had a threefold higher rate of succinate-dependent H₂O₂ production than that observed in dsr1 in the presence of ATP (Fig. 4A). H₂O₂ production in dsr1 mitochondria was reduced significantly compared with WT under normal growth conditions and after stress treatments (Fig. 4A).

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 H₂O₂ production from mitochondria. This finding is further supported by evidence that WT (JC66) seedlings simultaneously treated with catalase (for removal of H₂O₂) or either dicamba or SA had a dramatic decrease in promoter activity compared with the application of dicamba or SA alone (Fig. 4B).

To provide further support for these findings, we visualized the subcellular location of H₂O₂ 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. S8A). This staining showed that the majority of in vivo H₂O₂ 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 dsr1, these treatments increased H₂O₂ production (Fig. 4C), and this ROS originated from mitochondria, as seen in individual root protoplast images (Fig. S8A).

Given the difference in the sensitivity of dsr1 to foliar infection with *P. syringae* (Fig. 3C), we were interested in examining whether a biotic stress treatment of dsr1 resulted in altered H₂O₂ 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 H₂O₂ 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 H₂O₂ production in WT than in dsr1 (Fig. S8B and C). No staining was shown in either WT or dsr1 after injection with MgCl₂. Despite this decrease in H₂O₂ levels in dsr1, bacterial growth measurements did not show significant differences between dsr1 and WT over 3 d (Fig. S8D), 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-
mH2O2 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, mutagenically debilitating effects 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 mH2O2 production, it was still resistant to the avirulent bacteria Pst DC3000 (avrRpt2). The resistance may be due to other H2O2 production centers in the plant that were fully functional, thereby allowing H2O2 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 apoptotic 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 mH2O2 in dsr1.

Complex II Is a Source of mH2O2 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. NA. How oxidases are precisely 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 H2O2 (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 mH2O2 production is faster than pyruvate/malate-dependent mH2O2 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 H2O2 and SA as well as contradictory evidence as to whether SA acts upstream of H2O2 or vice versa (42). Although there are data that H2O2 induces SA
accumulation (43), SA also enhances H₂O₂ production (44), and H₂O₂ production can occur in the apoplast, mitochondria, and/or chloroplast (42, 45). Here, we have shown that GSTF8 requires mH₂O₂ 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 mutation broadens our understanding of SDH mechanisms and may provide insights into SDH-mediated mitochondrial perturbations through 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 dsrl as a loss-of-function mutant (nil or low LUC activity after hormone treatment). Gene expression in WT and dsrl was analyzed by quantitative RT-PCR, and microarrays were performed per a standard Affymetrix protocol. R. solani AGB was grown in culture, and seedlings sown in 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 dsrl by using Percoll density gradients. Respiration, enzyme assays, and H₂O₂ measurements were undertaken by using Clark-type O₂ electrodes, spectrophotometric and spectrfluorometric analysis of DCPiP reduction, Amplex Red oxidation, and H₂DCFDA 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.

**ACKNOWLEDGMENTS.** We thank Hayley Casarroto for technical assistance (CSIRO Plant Industry, Perth, Australia) and Drs. Kemal Kazan, Jeff Ellis, and David Day for critical comments on the manuscript. A.H.M. was supported by Australian Research Council Professorial Fellowship DP0771156. Research support from ARC Centre of Excellence in Plant Energy Biology Grant CE0561495 (to A.H.M.), CSIRO (K.B.S.), and the Grains Research and Development Corporation (K.B.S. and L.F.T.) are gratefully acknowledged.

Supporting Information

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SI Experimental Procedures

Bioluminescence Assays. Catalase (2,350 U/mL; Sigma), 10 mM malonate (pH 7; Sigma), 1 mM 2,6-dichloroisonicotinic acid (Aldrich), 1 mM benzoic acid (Sigma), 1 mM salicylic acid (SA; Sigma), 1 mM and 7 mM dicamba (Cadence, 700 g/kg, M₉₀ = 325 g/mol; Syngenta) were used as plant treatments. The treatments were poured onto 56-mm Petri dishes containing 4-d-old Arabidopsis seedlings. After 40 min of incubation at room temperature, the excess liquid was discarded, and the plates were transferred to the camera to be photographed such that, after acquiring the 0-h bioluminescence image, 1 h had elapsed. Bioluminescence was then measured every hour afterward for up to 20 h.

For the catalase experiments, the seedlings were treated for 40 min with treatments alone (equal volumes of water mixed with dicamba, SA, or catalase) or catalase treatments (equal volume of catalase mixed with SA or dicamba). Final concentrations were dicamba (3.5 mM), SA (1 mM), and catalase (2,350 U/mL). Luminescence was measured 7 h after treatment was initiated.

For the malonate pretreatment, JC66 seedlings were flooded for 2 h with either water (mock) or 10 mM malonate pretreatment. After the pretreatments, the seedlings were rinsed with water and subjected to an additional 40-min treatment with either dicamba (7 mM) or SA (1 mM). The average bioluminescence (light units) was measured per seedling at 4 h after this second treatment.

Mutant Screen and Mapping of Disrupted in Stress Responses 1 (dsr1). Approximately 100,000 M2 seedlings from ethyl methanesulphonate-mutagenized seeds of JC66 were screened. Mutants were classified as gain of function (constitutive GSTF8 promoter activity), enhanced function (increased promoter activity after treatment with SA), or loss of function [nil or low luciferase (LUC) activity after hormone treatment]. A total of 182 mutants fell into the above categories, and these were evaluated in their M3 generation to identify mutants for further analysis. Evaluation was based on a clear and robust LUC phenotype that mimicked the M2 phenotype and did not segregate for the mutant phenotype, had a recessive, monogenetic trait. DNA was extracted from plants in the selection process, 4 were loss of function. However, through analysis of F2 outcrossed populations, only one of these mutants had a mutation that was unlinked to the LUC transgene and displayed a monogenic trait. For mapping, we carried out a genetic cross between dsr1 (Columbia-0) and Landsberg plants. dsr1 was crossed with Landsberg erecta (Ler), and F2 analysis showed a 3:1 segregation for the mutant phenotype, indicating that the dsr1 mutation was a recessive, monogenic trait. DNA was extracted from plants exhibiting no LUC response to 1 mM SA or 7 mM dicamba treatment, and DNA from 20 to 40 homozygous dsr1 F2 plants was roughly mapped with a set of 18 simple sequence-length polymorphism (SSLP) markers to the bottom of chromosome 5, linked to marker ciw9. Additional mapping was performed by screening 244 F2 plants using SSLP and cleaved amplified polymorphic sequence (CAPS) markers near the marker ciw9. Because dicamba gave a more robust LUC response, a 7 mM dicamba treatment was used for the continued mapping. By first identifying plants that had no dicamba-induced LUC activity and then mapping with molecular markers in the region, dsr1 was mapped to a region between molecular markers SGCSNP18942 and SGCSNP404, spanning an ~370-kb interval. We used additional PCR markers to identify recombinants of the dsr1 region in a further 560 F2 plants. Only plants with recombination between the two markers were kept for seed, and the F3 seeds were tested for dicamba-induced LUC activity to ensure that these plants were homozygous for the dsr1 mutation. The F2 whose progeny had no herbicide-induced GSTF8 promoter activity were kept for further fine mapping, which narrowed down the dsr1 region to 29 kb, spanning the region between flanking CAPS markers CER434925 and CER440602. Within these markers were three BACs (K1F13, MSN2, and MUD21), spanning 50 genes. The dsr1 mutant was backcrossed to the WT (Columbia-0) three times, and the backcrossed plants had the same phenotype as the original dsr1 mutant.

Mapping Primers and Enzymes. The mapping primers and enzymes were as follows. SGCSNP18942: forward, 5′-ACACCGCAAC-CAAGTGAACA-3′ and reverse, 5′-AATTTGACAACTTGTGATTTTATCG-3′; aTagI digest. SGCSNP404: forward, 5′-TATCAAAAGCACTGACGTT-3′ and reverse, 5′-GATGTACTTGGACAGCACAAA-3′; BsaYI digest. CER434925: forward, 5′-GCCTCGTCGGTTGAGTTTA-3′ and reverse, 5′-AGTTCCGGTGGCTGATTT-3′; HindIII digest. CER440602: forward, 5′-CTTTTCGAGGAGCAGAGA-3′ and reverse, 5′-TTCCTAAAGGCGATCTGATAATG-3′; Apol digest.

Gene Expression Analysis by Quantitative RT-PCR. cDNA was generated from 1 μg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. The cDNA was used as the template in real-time quantitative PCR; the quantitative RT-PCR are similar to those described by Gao et al. (1). Fragments of interest were amplified by the Bio-Rad iCycler Real Time PCR machine using Bio-Rad SYBR green as the fluorescent dye. Relative gene expression was derived by using 2^ΔΔCT, where ΔΔCT represents CT of the gene of interest minus CT of the reference gene cyclophilin (ATCYP5). Where required, the significance of differences between relative gene expressions was analyzed by two-way ANOVA. Each experiment was performed in duplicate with three biological replicates.

The primers used were as follows. Cyclophilin: forward, 5′-TCTTTCCTTCTCGGAGGACCAT-3′ and reverse, 5′-AAGCTCGGAATTCTCGGAT-3′; TATA-1: forward, 5′-CGTCTCTCGGGCTTCTCTAGAGTACACC-3′ and reverse, 5′-CGAAGAACCTACCACTACCCAA-3′; SDH1-1: forward, 5′-CGTCTCTCGGCTGTGCTCTCCAGCCT-3′ and reverse, 5′-GCAGCAAACTATGTTGACCCTTGTG-3′; SDH1-2: forward, 5′-CGAAGAACCTACCCAGCCTTGTG-3′ and reverse, 5′-GCTGACACATGTGAGAAAGC-3′; GSTF8: forward, 5′-GCAGAAGAACCTACCACTACCCAA-3′ and reverse, 5′-CTTCTGAGAACCAGGAGA-3′; and the adjusted P value (Benjamini–Hochberg false discovery rate) for each array probe (2). Genes in SA-treated plants with significant changes in expression relative to the mock samples were identified by using an adjusted P value <0.05. The resulting list of genes was further reduced by selecting genes showing ≥1.5-fold change in expression. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession number GSE6883) (9, 10), and the complete list of genes, P values, and fold changes are presented in Supplemental Table 1.

**Isolation of Mitochondria Using Gradient Centrifugation.** Approximately 100 g of shoot material from 3-wk-old hydroponically grown Arabidopsis was homogenized with a Polytron blender (Kinematica) in 300 mL of cold grinding medium (0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 1% (wt/vol) polyvinylpyrrolidone 40, 2 mM EDTA, 10 mM KH₂PO₄, 1% (wt/vol) BSA, and 20 mM ascorbic acid (pH 7.5)) for 10 s, twice, with 5- to 10-s intervals between bursts. The homogenate was filtered through four layers of Miracloth and centrifuged at 1,500 × g for 5 min, and the resulting supernatant was then centrifuged at 24,000 × g for 15 min. The organelle pellet was washed by repeating the wash 1,500 and 24,000 × g centrifugation steps twice in sucrose wash medium. The resulting pellet of crude organelles was carefully resuspended in sucrose wash medium and gently layered over a 35-mL continuous 28% Percoll density gradient consisting of 0–4.4% polyvinylpyrrolidone 40. The gradient was then centrifuged at 40,000 × g for 45 min. The mitochondrial band was seen as a yellow-brown band near the bottom of the tube. The upper layers of the density gradient were removed, and the mitochondrial band was collected. The mitochondrial fraction was diluted approximately fivefold with sucrose wash buffer and centrifuged at 24,000 × g for 10 min. The wash fraction was further purified with a second Percoll density centrifugation as described for the first gradient. The final mitochondrial band was collected and washed three times by dilution and centrifugation.

**Succinate-Dependent Oxygen Consumption Using O₂ Electrode.** Oxygen consumption by purified mitochondria was measured by a computer-controlled Clark-type O₂ electrode unit (Hansatech Instruments). Calibration of the electrode was carried out by addition of sodium dithionite to remove all oxygen in the electrode chamber. The air-saturated oxygen concentration was assumed to be 240 μM at 25 °C for the following measurements. All reactions were carried out at 25 °C using 1 mL of mitochondrial reaction medium [0.3 M sucrose, 10 mM TES, 10 mM NaCl, 4 mM MgSO₄, and 0.1% (wt/vol) BSA (pH 7.2)] and ~100 μg of mitochondrial protein. To investigate the succinate-dependent O₂ consumption, 5 mM succinate was added to the reaction solution. To investigate the activation of succinate-dependent O₂ consumption by ATP, ATP was added to the reaction solution at a final concentration of 0.5 mM. NADH was also added to the reaction solution at a final concentration of 1 mM to measure total electron transport chain-linked O₂ consumption rate.

**Tissue Respiration Assay.** Oxygen consumption by root tissues (~80 mg) was measured by a computer-controlled Clark-type O₂ electrode unit as described above for O₂ consumption by isolated mitochondria, with the exception of no sucrose in the reaction medium.

**Succinate Dehydrogenase (SDH) Activity Assay Using Spectrophotometry.** The SDH activity was determined based on the method described in ref. 3, with the following modifications. The mitochondrial fraction (~30 μg of protein) was spectrophotometrically assayed for activity at 600 nm at 30 °C, in 1 mL of a reaction medium [50 mM potassium phosphate (pH 7.4), 10 mM sodium succinate, 0.1 mM EDTA, 0.1% (wt/vol) BSA, 10 mM potassium cyanide, 0.12 mM dichlorophenolindophenol (DCPIP), and 1.6 mM phenazine methosulfate]. For ATP activation experiments, the ATP was added into the 1-mL reaction solution to a final concentration of 0.5 mM. An extinction coefficient of 21 mM⁻¹·cm⁻¹ at 600 nm for DCPIP was used for calculations.

**Blue Native (BN) Gel, Mitochondrial Complex II (Complex II) Activity Staining, Bound FAD Analysis, and Differential In-Gel Electrophoresis (DIGE) Analysis.** The methods for BN gel electrophoresis and Complex II activity staining were adapted from methods described in refs. 4 and 5, with the following modifications. Mitochondria aliquots (1 mg of protein) were centrifuged for 10 min at 14,300 × g, and pellets were resuspended in the BN re-suspension buffer (pH 7.4, 30 mM Heps, 150 mM potassium acetate, and 10% glycerol) with the detergent digitonin (5 g of protein) and incubated on ice for 20 min. After centrifugation at 18,300 × g for 20 min, the supernatants were transferred into a new Eppendorf tube and 1 μL of 5% Serva Blue G solution (750 mM caproic amino acid and 5% Coomassie blue G-250) was added. After mixing, the solutions were transferred onto the gel. The 1D BN gel electrophoresis and 2D SDS/PAGE were carried out according to the methods in ref. 4. For Complex II activity staining, the 1D BN gels were soaked in staining medium [50 mM potassium phosphate buffer (pH 7.5), 84 mM succinate Na, 0.2 mM phenazine methosulfate, 2 mg/mL nitrotetrazolium blue, 4.5 mM EDTA, and 10 mM potassium cyanide] as described in refs. 5 and 6. After 2–3 h of staining, the gels were scanned. For the analysis of bound FAD, the method from ref. 7 was used. Briefly, SDS/PAGE gels were incubated for 20 min in 10% acetic acid to lower pH, and fluorescence emission at 526 nm by covalently bound FAD was analyzed after excitation at 488 nm in a Typhoon Trio imager (GE Healthcare). SDH I is the only major flavin-containing protein that is observed in plant mitochondria when using this technique of differential (before and after) 10% acetic acid staining visualization. DIGE analysis of changes in the mitochondrial proteome in isoelectric focusing SDS/PAGE gels using Cy dyes was conducted in triplicate per the methods described in ref. 8. Mass spectrometry identification of SDH I-1 was also conducted as outlined in ref. 8.

**H₂O₂ Production Assay by Isolated Mitochondria.** The rate of H₂O₂ production by isolated mitochondria was determined with the oxidation of the fluorogenic indicator Amplex Red in the presence of horseradish peroxidase (9). The concentrations of horseradish peroxidase and Amplex Red in the incubation medium (the medium used for O₂ consumption by mitochondria outlined above) were 0.1 unit/mL and 50 μM, respectively. Fluorescence was recorded in a microplate reader (Cary Eclipse) with 530-nm excitation and 590-nm emission wavelengths. In a typical experiment, mitochondria were incubated at 0.1 mg of protein per mL at 25 °C for 60 min. H₂O₂ production by isolated mitochondria was initiated by adding succinate (5 mM) as substrate.

**Detection of H₂O₂ Production in Isolated Root Protoplasts with H₂ Dichlorofluorescein Diacetate (DCFDA).** The roots from 2-wk-old hydroponic plants were cut and transferred into hydroponic solution with addition of 0.5 M sorbitol, 0.05 M sucrose, 0.3% cellulose (Onozuka R-10), 0.1% pectinase (Macerozyme R-10), and 0.1% BSA. After a 3-h digestion at room temperature in the dark, the solution was filtered with nylon cloth (70 μm) and centrifuged at 350 × g for 6 min at room temperature. Pellets were washed with the same solution without cellulose and pectinase (washing solution) and then centrifuged at 350 × g for 6 min at room temperature. The pellets containing isolated protoplasts were resuspended in 2 mL of washing solution. Aliquots of protoplasts were treated with 100 μM SA or dicamba for 30 min and then stained with 5 μM H₂DCFDA and 200 mM MitoTracker Red CMXRos for 10 min. The protoplasts were subjected to fluorescence microscopy for observation. The H₂O₂ production using DCFDA staining was recorded based on light intensity, and average light intensity per protoplast was measured with image analysis software.
Metabolites were extracted from frozen Arabidopsis leaves according to a method adapted from that described in ref. 10, and derivatized metabolite samples were analyzed on an Agilent Technologies GC/MSD system. Raw GC-MS data preprocessing and statistical analysis were performed with MetabolomeExpress software (version 1.0; http://www.metabolome-express.org). Detailed methods are given in ref. 11.

Rhizoctonia solani Bioassays. The source of R. solani anastomosis group 8 (AG8) strain, its maintenance, and bioluminescence techniques have been described previously (12). *Rhizoctonia* infections were performed as described in ref. 13. Briefly, R. solani AG8 was grown on potato dextrose agar plates (4 d at 21 °C). Potato dextrose broth was inoculated with mycelia, and the culture was grown for 7 d at 21 °C with slow (35 rpm) shaking. Mycelium was collected by filtration, homogenized in 100 mL of sterile water, pelleted by centrifugation, and resuspended in sterile water to 1 × 10^7 mycelium pieces-mL^-1. Seedlings were sown in vermiculite and inoculated with 1 mL of mycelium. Each pot contained four seedlings, and five replicate pots were planted for each genotype. Individual pots were covered with clear plastic domes and transferred to a growth chamber (22 °C, 16 h of light per day). Survivorship rates were scored at 4 and 14 d after infection.

**P. syringae Bioassays.** The bacterial strains used were *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) and Pst DC3000 (avrRpt2). Plant leaves were syringe infiltrated with 5 × 10^6 cells per mL, and bacterial enumeration was performed by using standard methods and repeated in independent experiments (14, 15).

**Alternaria Bioassays.** *Alternaria brassicicola* assays were performed with isolate UQ4273 according to the methods of Schenk et al. (16), with the following modifications. *A. brassicicola* was grown on half-strength potato dextrose agar, and the spores were scraped off and resuspended in half-strength potato dextrose broth to a concentration of 5 × 10^8 spores per mL. Leaves were inoculated with a 5-μl drop of spore suspension, and plants were incubated at 27 °C (16 h light/8 h dark). Lesion size was measured with the ImageJ freeware package (http://rsb.info.nih.gov/ij/).

**Evaluation of Salt and Herbicide Tolerance.** Surface-sterilized *Arabidopsis* seeds were sown on Murashige and Skoog media supplemented with increasing concentrations of NaCl, mannitol, or the herbicide dicamba. The concentrated dicamba stock (7 mM) was diluted in water before addition to the media. For the dicamba and NaCl experiments, JC66 (WT) and *dsr1* seeds were surface-sterilized, and plates were sown containing increasing concentrations of the salt or herbicide (concentrations are indicated in Fig. S2). The seeds were stratified for 3 d at 4 °C and then placed vertically in a 21 °C growth room with a 16-h light/8-h dark cycle. At 5 d after seeds were placed in the growth room, the plants were removed from the agar medium, and the length of the primary root was measured. There were two replicate plates of 10 plants for each herbicide and salt concentration tested, and the experiment was repeated at least twice. For the mannitol experiments, 4-d-old JC66 and *dsr1* seedlings were transferred to Murashige and Skoog media with 0, 100, 200, and 300 mM mannitol. The position of the root tip was marked, and new growth was measured after 8 d on the salt medium.

**Staining for H_2O_2 by 3,3′-Diaminobenzidine (DAB).** Leaves of 4-wk-old Arabidopsis seedlings were injected with either Pst DC3000 (avrRpt2) at a concentration of 5 × 10^7 cfu/mL or 10 mM MgCl_2, and plants were placed into the growth chamber at 22 °C under conditions of 14-h days and 10-h nights. At 17 h (7 h light, 10 h dark) after injection, H_2O_2 was visualized by DAB staining (17). The injected leaves were collected and incubated in 0.1% (wt/vol) DAB overnight on an orbital shaker. The leaves were boiled in 96% (vol/vol) ethanol for 10–20 min to remove the chlorophyll. Quantiﬁcation of the DAB staining was performed with ImageJ software. The average index of staining for each leaf (*n = 70*) was measured in the injected area minus the average on the opposite side of the leaf. The experiment was repeated with similar results.

Fig. S1. GSTF8 promoter activity in WT and dsr1 seedlings after chemical treatments with 1 mM benzoic acid (BA) and 1 mM 2,6-dichloroisonicotinic acid (INA). Bioluminescence levels are shown in the logarithmic scale. Average of 10 seedlings (± SEM).
Fig. S2. dsr1 plants and evaluation of their tolerance to abiotic stresses. (A) Representative 2.5-wk-old plants of WT and dsr1 (Scale bar: 1 cm.) (B and C) Measure of root elongation in WT and dsr1 seedlings on Murashige and Skoog media supplemented with increasing concentrations of NaCl (B) or dicamba (C). (D) Determination of the effect of increasing concentrations of mannitol on root elongation in WT and dsr1 seedlings, represented as the percentage root growth compared with that of the control (root growth on media with no mannitol supplement). Experiments in B–D contain an average of 20 seedlings (± SE). *Differences by least-square means Tukey’s honestly significant difference test (P < 0.05). Experiments were repeated twice with similar results.
Fig. S3. Differential gene expression in dsr1 plants. (A) Venn diagrams of probe identities from the Affymetrix ATH1 microarray that were significantly (≥2-fold change) regulated upon a mock (water) or SA treatment in dsr1 compared with WT. (B) Comparison of the expression profiles of the 100 most SA-induced genes in WT to their expression profiles in SA-treated dsr1. The 18 genes that are induced in WT but show significant repression in dsr1 are highlighted in red.
Fig. S4. Response of dsr1 to foliar bacterial and fungal pathogens. (A) Log bacterial counts from leaf tissue after Pst DC3000 infection of WT and dsr1 over 3 d. (Error bars: SEM; *P < 0.05.) (B and C) The lesion size on intact plants was measured at 3 d after inoculation with the fungus A. brassicicola (B) with representative leaves shown (C) (n = 10). Asterisks indicate values that are significantly different from each other (**P < 0.01, Student's t test.) Experiment was repeated twice with similar results.
Conservation of SDH1 and site of mutation in Saccharomyces cerevisiae, Chlamydomonas reinhardtii, and Homo sapiens showing wide conservation of amino acid 581 (highlighted in red), which is converted from Ala to Thr in dsr1. ClustalW alignment is shown, where asterisks (*) mean that the residues are identical, colons (:) indicate conserved substitutions, and periods (.) mean semiconserved substitutions. (Right) Model of the 3D structure of Complex II (comprising SDH1, -2, -3, and -4) adapted from Sun et al. (1) (http://molvis.sdsc.edu/fgij/fg.htm?mol=1ZOY). The mutation site is marked in yellow.

### Fig. 55. Conservation of SDH1 and site of mutation in dsr1

**Left** Protein sequence alignment of SDH1 from Arabidopsis thaliana, Chlamydomonas reinhardtii, Saccharomyces cerevisiae, and Homo sapiens showing wide conservation of amino acid 581 (highlighted in red), which is converted from Ala to Thr in dsr1. ClustalW alignment is shown, where asterisks (*) mean that the residues are identical, colons (:) indicate conserved substitutions, and periods (.) mean semiconserved substitutions. (Right) Model of the 3D structure of Complex II (comprising SDH1, -2, -3, and -4) adapted from Sun et al. (1) (http://molvis.sdsc.edu/fgij/fg.htm?mol=1ZOY). The mutation site is marked in yellow.

Fig. S6. Characteristics of complemented lines. (A) LUC response of GSTF8 promoter in dsr1 complementation (LC) lines. The coding region of SDH1-1 was PCR amplified and inserted behind a 35S promoter of the vector pEarlygate100 and introduced into dsr1 plants by Agrobacterium tumefaciens-mediated transformation. Average light units per seedling of WT, dsr1, and the complemented lines (LC1–LC9) at 10 h posttreatment with dicamba are shown. LC1–LC3 lines are T3, and LC4–LC9 lines are T2. Experiments are representative of three experiments (±SEM). (B) Gene expression of SDH1 -1 in dsr1 complementation (LC) lines. Relative expression of SDH1-1 in 4-d-old untreated and 7 mM dicamba-treated seedlings was measured for two independent T3 lines. Experiments are representative of three experiments, each containing two technical and three biological replicates (±SEM). (C) Percentage pollen viability in WT and dsr1 plants. Pollen (n = 400) from flowers of at least two plants was stained with Alexander’s stain and scored for viability. (D) Expression of SDH alleles in WT and dsr1. Relative expression of SDH1-1 and SDH1-2 in WT and dsr1 plants at 10 h after a 40-min treatment with water (mock), 7 mM dicamba, or 1 mM SA. Experiment is representative of three experiments, each containing two technical and three biological replicates (±SEM).
Fig. S7. SDH abundance and Complex II composition in WT, complemented line, and dsr1. (A) Complex II abundance and FAD bound to SDH1 in WT, dsr1 complemented line, and dsr1. The mitochondria were solubilized with digitonin (5.0 g/g) for BN-PAGE. (B) BN in-gel activity staining and 2D resolution of digitonin-solubilized (5 g/g) mitochondrial protein complexes from dsr1 and WT by BN-SDS/PAGE. The region in the dashed boxes contained subunits of Complex II according to previous reports (1, 2). (C) DIGE on 2D isoelectric focusing SDS/PAGE gels. Mitochondrial proteins samples from WT (labeled with Cy3; red) and dsr1 (labeled with Cy5; green) and samples from a complemented line (labeled with Cy3; red) and WT (labeled with Cy5; green) were compared, respectively. The yellow spots represent proteins of equal abundance between WT and dsr1 and the complemented line. The yellow spot circled with white was identified as SDH1-1 (At5g66760) with specific peptides distinct from SDH1-2 (At2g18450). Peptides [ion scores >37 (shown in parentheses); P < 0.05] specific to SDH1-1 were AFGGQSLDFGK (57), SSQTLATGGYGR (76), IMQNNAAVFR (39), and HTLGYWEDEK (80).

Fig. S8. (A) The distribution of H$_2$O$_2$ production in WT and dsr1 protoplasts in response to chemical treatments shows the mitochondrial origin of DCF fluorescence. Representative microscopy images of Arabidopsis WT root protoplast H$_2$O$_2$ production by DCF fluorescence, counterstaining by MitoTracker Red, and image overlay for negative control, 100 μM SA, and 100 μM dicamba treatments for 30 min. Although these images show the origin of the signals in the cells, differences between the intensity of DCF staining between treatments cannot be assessed from these data. To masses differences, the average of many cells is needed, and those data are provided and statistically assessed in Fig. 4C. (B–D) H$_2$O$_2$ production is reduced in dsr1 leaves infiltrated with Pst DC3000 (avrRpt2). (B) DAB staining of leaf tissue from WT and dsr1 infected with Pst DC3000 (avrRpt2). (C) Average leaf DAB staining intensity in infected zone. (D) Log bacterial counts from leaf tissue after Pst DC3000 (avrRpt2) infection of WT and dsr1 over 3 d. (Bars: SEM; *P < 0.0001, Student’s t test).

Other Supporting Information Files

Dataset S1 (XLS)