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

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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 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.

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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)], auxin herbicides (e.g., dicamba), and ROS (H₂O₂) 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 (JCC6) 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 JCC6 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 (JCC6) plants in response to SA (Fig. 14). 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. 14). 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. 14).
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. L4). A decrease in both SA and dicamba-induced GSTF8 transcriptional levels in dsr1 compared with WT confirmed the LUC results (Fig. 1B). In contrast, the induction of the GSTF8 promoter activity by H2O2 seen in WT seedlings also occurred in dsr1 (Fig. L4), 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. L4). 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 S1d).

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 S1f).

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.

**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.]

**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. S4B). To determine whether dsr1 was also susceptible to a 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. 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 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. S6C). 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. 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 dichloro-phenolindophenol (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.

![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 & S, 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.)](image)

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
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 (IC66) seedlings simultaneously treated with catalase (for removal of H2O2) and 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 localization of H2O2 production in Arabidopsis roots by using dichlorofluorescin 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. S84).

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. S8 B 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-
Plant mitochondria have been previously implicated in the production of mROS, and of the leaf, Syringa vulgaris, Pst DC3000 (avrRpt2) plants did not exhibit altered pollen development (Fig. S6C) 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 mH2O2 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. 5E), suggests that a post-transcriptional 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 strong 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 AS81T 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 vitro in 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 mH2O2 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 mH2O2 production was also increased by ATP in WT but not dsr1, again implicating the substrate-binding pocket in modulating H2O2 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.

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 Arabidopsis with harpin or avirulent Pst were reported to cause the production of mROS, change the membrane potential, and inhibit ATP synthesis (29, 30). However, mutagenesis of the 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 could 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 mutants lacking apoplastic ROS production from SDH1-1 were reported to cause the production of mROS, 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. NADPH oxidases are typically the primary ROS producers 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 H$_2$O$_2$ production (44), and H$_2$O$_2$ production can occur in the apoplast, mitochondria, and/or chloroplast (42, 45). Here, we have shown that GSTF8 requires mH$_2$O$_2$ for its gene expression and that SA can be translated into an SDH1-dependent ROS signal by the mitochondrion 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 AGB was grown in culture, and seedlings sown into vermiculite were inoculated by watering. A. brassicicola spore suspension was grown on leaf-discs and dsr1 by using Percoll density gradients. Respiration, enzyme assays, and H$_2$O$_2$ measurements were undertaken by using Clark-type O$_2$ electrodes, spectrophotometric and spectrophotometric analysis of DCP1P 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.metabolomeexpress.org).

Further detailed methods are available in SI Experimental Procedures.

ACKNOWLEDGMENTS. We thank Hayley Casarotto 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.