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Research

Succinate dehydrogenase (mitochondrial complex II) is a source of reactive oxygen species in plants and regulates development and stress responses

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Summary

- Reactive oxygen species (ROS) are signaling molecules that regulate plant development and responses to stresses. Mitochondria are the source of most ROS in heterotrophic cells, and mitochondrial complex I and complex III are regarded as the main sites of ROS production in plant mitochondria. Recent studies have demonstrated that succinate dehydrogenase (SDH) also contributes to mitochondrial ROS production. However, the ability of SDH to generate ROS in plants is unclear. The aim of this study was to evaluate the role of SDH in mitochondrial ROS production.
- Our results demonstrated that SDH is a direct source of ROS in *Arabidopsis thaliana* and *Oryza sativa*, and the induction of ROS production by specific SDH inhibitors impaired plant growth. In addition, this effect was accompanied by the down-regulation of cell cycle genes and the up-regulation of stress-related genes.
- However, the partial inhibition of SDH by a competitive inhibitor decreased ROS production, which was associated with increased shoot and root growth, and prevented the down-regulation of cell cycle genes and the induction of stress-related genes by noncompetitive inhibitors.
- In conclusion, SDH plays an important role in ROS production, being a direct source of ROS in plant mitochondria and regulating plant development and stress responses.

Introduction

Succinate dehydrogenase (SDH; succinate: ubiquinone oxidoreductase; mitochondrial complex II) plays a central role in mitochondrial metabolism, catalyzing the oxidation of succinate to fumarate and the reduction of ubiquinone (UQ) to ubiquinol (UQH_2), thereby linking the tricarboxylic acid (TCA) cycle and the electron transport system (ETS). Classically, SDH is composed of four subunits, named SDHA–SDHD in *Escherichia coli* and animals and SDH1–SDH4 in yeast and plants. SDHA/SDH1 is a flavoprotein subunit that has a dicarboxylate (succinate) binding site and a flavin adenine dinucleotide (FAD) cofactor. SDHB/SDH2 is an iron–sulfur protein subunit that contains three Fe–S clusters. The two other hydrophobic, membrane-anchored subunits, SDHC/SDH3 and SDHD/SDH4, contain the UQ binding site (Q-site) (Yankovskaya *et al.*, 2003).

In eukaryotic cells, the ETS drives the majority of ATP synthesis, but it is also a major source of reactive oxygen species (ROS). Complex I (NADH: ubiquinone oxidoreductase) and complex III (UQH_2: cytochrome c oxidoreductase; cytochrome bc1 complex) are generally regarded as the main sources of ROS production (Kowaltowski *et al.*, 2009; Murphy, 2009; Brand, 2010; Møller & Sweetlove, 2010). Nevertheless, structural analyses of SDH suggested that this enzyme can produce ROS at the FAD-binding site through the monovalent electron reduction of O_2 (Yankovskaya *et al.*, 2003).

Recently, SDH was unequivocally demonstrated to be an important and direct source of ROS in mammals (Quinlan *et al.*, 2012). In addition, SDH has been recognized as an indirect modulator of superoxide production by complexes I and III (Boveris *et al.*, 1972; Møller & Sweetlove, 2010; Dröse *et al.*, 2011; Bleier & Dröse, 2013). However, in plants, the direct contribution of SDH to mitochondrial ROS production has not yet been established.

In plants, mutations in SDH subunits were associated with changes in development and in ROS homeostasis. Heterozygous *SDH1-1/sdh1-1 Arabidopsis thaliana* showed low SDH activity but increased photosynthesis, nitrogen assimilation and stomatal conductance (Fuentes *et al.*, 2011). However, pollen abortion and reduced seed set were also reported when SDH1-1 levels

were decreased by RNA interference (León *et al.*, 2007). In addition, *A. thaliana* possess an *SDH1-2* gene that is significantly expressed only in roots, and homozygous mutations in this gene do not affect growth or development (León *et al.*, 2007).

In *Solanum lycopersicum*, RNA interference against SDH2-2 decreased SDH activity and increased the rate of photosynthesis, stomatal opening and plant growth (Araújo *et al.*, 2011). Metabolic alterations in organic acid concentrations caused by decreased SDH activity have been suggested to be responsible for these changes in plant development, but the role of SDH as a source of ROS production in plant mitochondria has yet to be considered. In another study, a point mutation in the substrate-binding site of SDH1-1 (mutant *disrupted stress response 1* (*dsr1*)) reduced SDH activity and decreased mitochondrial ROS production in *A. thaliana* (Gleason *et al.*, 2011), but direct ROS formation at the level of the SDH-1 subunits was not tested. In addition, this mutation impairs the salicylic acid (SA)-induced expression of stress-related genes, indicating an important role of SDH-derived ROS in regulating the expression of plant defense genes.

In view of the central role of SDH in mitochondrial metabolism and the importance of ROS in signaling processes, the aim of this study was to evaluate the capacity of SDH to generate ROS in plant mitochondria and to regulate plant development and stress-related gene expression. Our results demonstrated that SDH is a direct source of ROS in isolated mitochondria and that the ROS production rate is proportional to the reduced state of the flavin group of the SDH1 subunit, consistent with the hypothesis that SDH has a similarly direct role in mitochondrial ROS generation in plants as in mammals (Quinlan *et al.*, 2012). The induction of ROS production by noncompetitive SDH inhibitors impaired plant growth without inhibiting mitochondrial oxygen consumption, indicating that this effect is independent of SDH activity and could instead depend on ROS signaling. In addition, this effect was accompanied by the down-regulation of cell cycle genes and the up-regulation of stress-related genes. However, partial SDH inhibition using the competitive inhibitor malonate (MA) decreased hydrogen peroxide (H_2O_2) production and was associated with increased growth of shoots and roots, while impairing the induction of stress-related genes. Our results indicate that SDH is a source of ROS production in plants and that SDH modulates different cellular signaling processes, as well as cell cycle and stress responses, that are essential to normal plant development.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. seeds were germinated in half-strength Murashige & Skoog (MS) medium at 14°C with a 16-h photoperiod. Two weeks after being sown, the *A. thaliana* seedlings were transferred to hydropony in 200-ml plastic cups (three seedlings per cup) filled with 10% MS medium solution. Rice (*Oryza sativa* L. Nipponbare) seeds were germinated in MS medium (Sigma-Aldrich) at 25°C with a 12-h photoperiod. One week after being sown, the rice seedlings were transferred to hydropony in 200-ml plastic cups (three seedlings per cup) filled with Hoagland–Arnon's nutritive solution (Hoagland & Arnon, 1950).

Isolation of mitochondria using a self-generated Percoll gradient

Mitochondria were isolated from the roots of 2-wk-old plants as previously described by Neuburger *et al.* (1982), with some modifications, such as extraction buffer containing 10 mM HEPES/Tris, pH 7.4, 0.3 M mannitol, 2 mM EGTA, 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 20 mM β-mercaptoethanol, and 0.1% (w/v) fatty acid-free bovine serum albumin (fat-free BSA). The final protein concentration varied from 10 to 20 mg ml^{-1}.

Isolation of protoplasts

Protoplast isolation was performed as described by Chen *et al.* (2006). Protoplast transformation was performed as described by Tao *et al.* (2002). After transformation, protoplasts were incubated for 24–48 h in the dark at 28°C before imaging. Fluorescence was monitored on an Olympus FluoView 1000 confocal laser scanning microscope (Olympus, Japan) equipped with a set of filters capable of distinguishing between green and yellow fluorescent protein (GFP and YFP, respectively) and plastid autofluorescence. The images were captured with a high-sensitivity photomulitiplier tube detector.

$FADH_2/FAD^+$ status measurement

The $FADH_2/FAD^+$ redox state was determined based on autofluorescence using a fluorimeter with (excitation/emission wavelength) Ex/Em = 490 ± 10 nm/530 ± 10 nm (Kunz & Gellerich, 1993). The assay was performed using coupled isolated mitochondria (0.5 mg ml^{-1}) in respiration medium (see the 'Oxygen consumption measurement' subsection). The additions are indicated in the figure legends.

Measurement of SDH activity

The activity of SDH was determined spectrophotometrically using 2,6-dichlorophenol-indophenol (DCPIP) as an artificial electron acceptor and succinate as the substrate (Robinson & Lemire, 1995). The assay was performed at room temperature (25°C) in 1.0 ml of reaction medium containing 20 mM phosphate buffer, pH 7.2, 0.1% Triton X-100, 4 mM sodium azide, and 50 μM DCPIP. Experiments using coupled isolated mitochondria were carried out in 1.0 ml of respiration medium (see the 'Oxygen consumption measurement' subsection) supplemented with 4 mM sodium azide and 50 μM DCPIP.

Blanks were obtained in the absence of succinate. The reaction was started by adding 10 mM succinate using 0.1 mg ml^{-1} of the final protein concentration. The reduction of DCPIP was monitored for 10 min at 600 nm. SDH activity was calculated using

the molar absorption coefficient of reduced DCPIP ($21.0\ mM^{-1}\ cm^{-1}$).

Oxygen consumption measurements

Oxygen consumption rates were measured polarographically using high-resolution respirometry (Oroboros Oxygraph-O_2K; Oroboros Instruments, Innsbruck, Austria). The electrode was calibrated between 0 and 100% saturation with atmospheric oxygen at 28°C. The isolated mitochondria or rice protoplasts ($0.2\ mg\ ml^{-1}$) were incubated with 2.0 m; of the standard respiration buffer containing 0.3 M mannitol, 10 mM Tris-HCl, pH 7.2, 3 mM $MgSO_4$, 10 mM NaCl, 5 mM KH_2PO_4, 0.3 mM β-NAD^+, and 0.1% (v: v) fat-free BSA.

Determination of mitochondrial H_2O_2 release

H_2O_2 release was measured using the Ampliflu Red (Sigma-Aldrich) oxidation method as previously described (Smith *et al.*, 2004). Briefly, mitochondria or protoplasts ($0.2\ mg\ protein\ ml^{-1}$) were incubated in standard respiration buffer (see the 'Oxygen consumption measurement' subsection) or 10 mM MES, pH 6.5, respectively, supplemented with 10 mM Ampliflu Red and $5\ units\ ml^{-1}$ horseradish peroxidase. Fluorescence was monitored using a fluorimeter at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5 nm), respectively. Calibration was performed by the addition of known quantities of H_2O_2.

H_2O_2 release was evaluated in rice or *A. thaliana* tissues using 3,3-diaminobenzidine (DAB) staining. Plant tissue was incubated overnight in DAB staining solution ($1\ mg\ ml^{-1}$ DAB in 10 mM MES, pH 6.5) and photographed by digital photography.

Mitochondrial membrane potential ($\Delta\psi_m$) determination

The $\Delta\psi_m$ was measured using the fluorescence signal of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria (Akerman & Wikstron, 1976). Isolated mitochondria ($0.2\ mg\ protein\ ml^{-1}$) were incubated in standard respiration buffer (see the 'Oxygen consumption measurement' subsection) supplemented with 15 mM safranine O. Two millimolar carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) was used as a positive control to collapse $\Delta\psi_m$. Fluorescence was detected at an excitation wavelength of 495 nm (slit 5 nm) and an emission wavelength of 586 nm (slit 5 nm). Data were reported in arbitrary fluorescence units. Other additions are indicated in the figure legends.

Quantitative real-time PCR (RT-qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen, Waltham, MA, USA). Complementary (c)DNA was obtained using the SuperscriptTMII (Life Technologies, Carlsbad, CA, USA) reverse transcriptase system and a 24-polyTV primer (Invitrogen). After synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR reactions. All reactions were repeated four times, and expression data analyses were performed after comparative quantification of the amplified products using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). RT-qPCR reactions were performed in an Applied Biosystems StepOne plus Real Time PCR system (Applied Biosystems, Waltham, MA, USA) using SYBR-green intercalating dye for fluorescence detection. The primer sequences and reference genes are listed in Supporting Information Table S1.

Microarray analysis

Microarray data were obtained from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo; accession no. GSE22942) (Gleason *et al.*, 2011).

Gene ontology analysis

Gene ontology analyses were performed using the web-based tool and database agriGO (Du *et al.*, 2010).

Functional protein association networks

The functional protein association network was created using the STRING database (Snel *et al.*, 2000) and analyzed using MEDUSA (Hooper & Bork, 2005) and VIACOMPLEX software (Castro *et al.*, 2009).

Protein determination

Protein concentrations were determined as described by Bradford (1976) using BSA as a standard.

Statistical analysis

Data were plotted with GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and analyzed by one-way ANOVA and *a posteriori* Tukey's test. *P*-values of 0.05 were considered statistically significant.

Results

The noncompetitive inhibition of SDH increases ROS production in plant mitochondria

Previous studies in mammalian mitochondria demonstrate that SDH is a site of ROS production and that this pathway is induced by inhibiting SDH using noncompetitive inhibitors (Chen *et al.*, 2007; Quinlan *et al.*, 2012). To verify whether noncompetitive inhibition of SDH is also able to induce ROS production in plants, leaf and root tissues from *A. thaliana* were treated with 10 μM thenoyltrifluoroacetone (TTFA), and the H_2O_2 content was evaluated by histological staining. DAB staining showed that TTFA induced H_2O_2 production in both leaf and root tissues (Fig. 1a), and the increase of ROS production in leaves treated with TTFA was confirmed by dichlorofluorescein

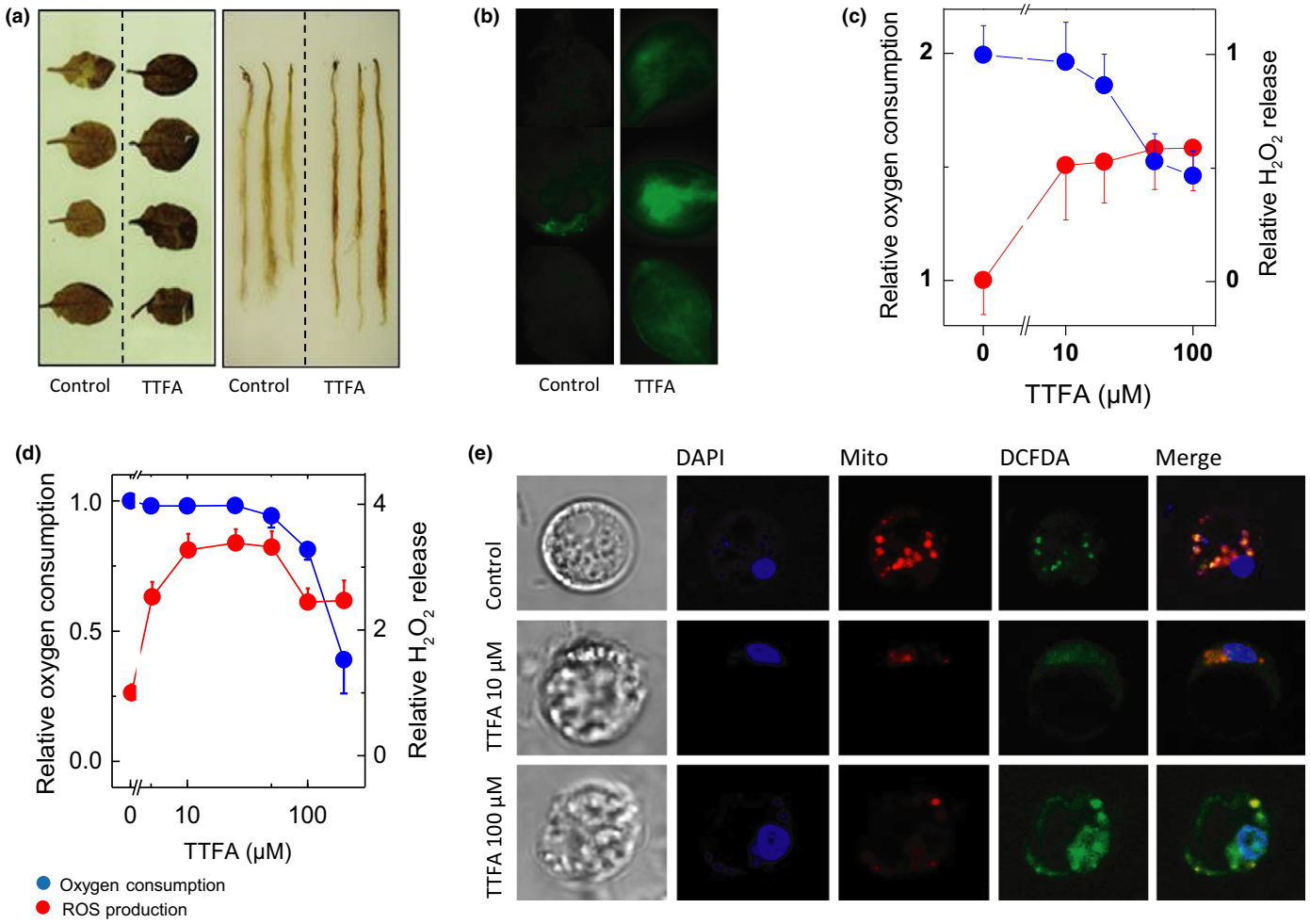

Fig. 1 TTFA induces ROS production in *Arabidopsis thaliana*. (a) 3,3-Diaminobenzidine (DAB) staining indicating reactive oxygen species (ROS) production in tissues treated with 10 μM thenoyltrifluoroacetone (TTFA). (b) Evaluation of succinate dehydrogenase (SDH)-dependent ROS production by dichlorofluorescein diacetate (DCFDA) fluorescence in *A. thaliana* leaves. (c) The effect of increasing concentrations of TTFA on hydrogen peroxide (H_2O_2) release (red) and on oxygen consumption (blue) in *A. thaliana* roots was also quantified fluorometrically. (d) ROS production and SDH-dependent oxygen consumption were also measured in isolated protoplasts in the presence of increasing concentrations of TTFA and confirmed by confocal microscopy of protoplasts treated with 10 and 100 μM TTFA (e). The numeric values represent the mean ± SE of three independent experiments. DAPI, 4′,6-diamidino-2-phenylindole; Mito, mitotracker.

diacetate (DCFDA) staining (Fig. 1b). In addition, the TTFA effect on leaf H_2O_2 production was measured fluorometrically in parallel with oxygen consumption using increasing concentrations of TTFA. Interestingly, a low dose of TTFA was able to induce ROS generation without inhibiting oxygen consumption (Fig. 1c). A similar result was also observed in rice, as shown in the Supporting Information (Fig. S1).

The effect of TTFA on mitochondrial ROS production and oxygen consumption was also confirmed in isolated protoplasts. Noncompetitive SDH inhibition by TTFA was followed by a decrease in succinate-dependent oxygen consumption and an approximately three-fold increase in ROS production (Fig. 1d). As verified in intact leaves, low doses of TTFA were able to increase ROS production without inhibiting oxygen consumption. In addition, confocal microscopy demonstrated that TTFA treatment increased cellular ROS content (Fig. 1e). These results demonstrate that TTFA is able to induce ROS production in plant mitochondria and suggest that, because of its specificity, SDH can be a source of ROS generation, as described in mammalian cells (Quinlan *et al.*, 2012).

To determine the role of SDH in ROS production pathways, the effect of competitive and noncompetitive SDH inhibition on different bioenergetic parameters was evaluated using isolated mitochondria. In the presence of succinate, the substrate of SDH, the competitive inhibitors MA, 3-nitropropionic acid (3NP) and oxaloacetate (OAA) increased the oxidative state of the SDH flavin group, whereas the noncompetitive SDH inhibitors (Q-site inhibitors) TFFA and SA inhibited electron delivery from SDH and increased the reduced state of the flavin group (Fig. 2a). Only the competitive inhibitors blocked succinate oxidation by SDH (Fig. 2b), demonstrating that these inhibitors target the SDH1 subunit. However, all the inhibitors decreased succinate-induced oxygen consumption (Fig. 2c), demonstrating that both types of SDH inhibitors impaired electron transport from succinate to

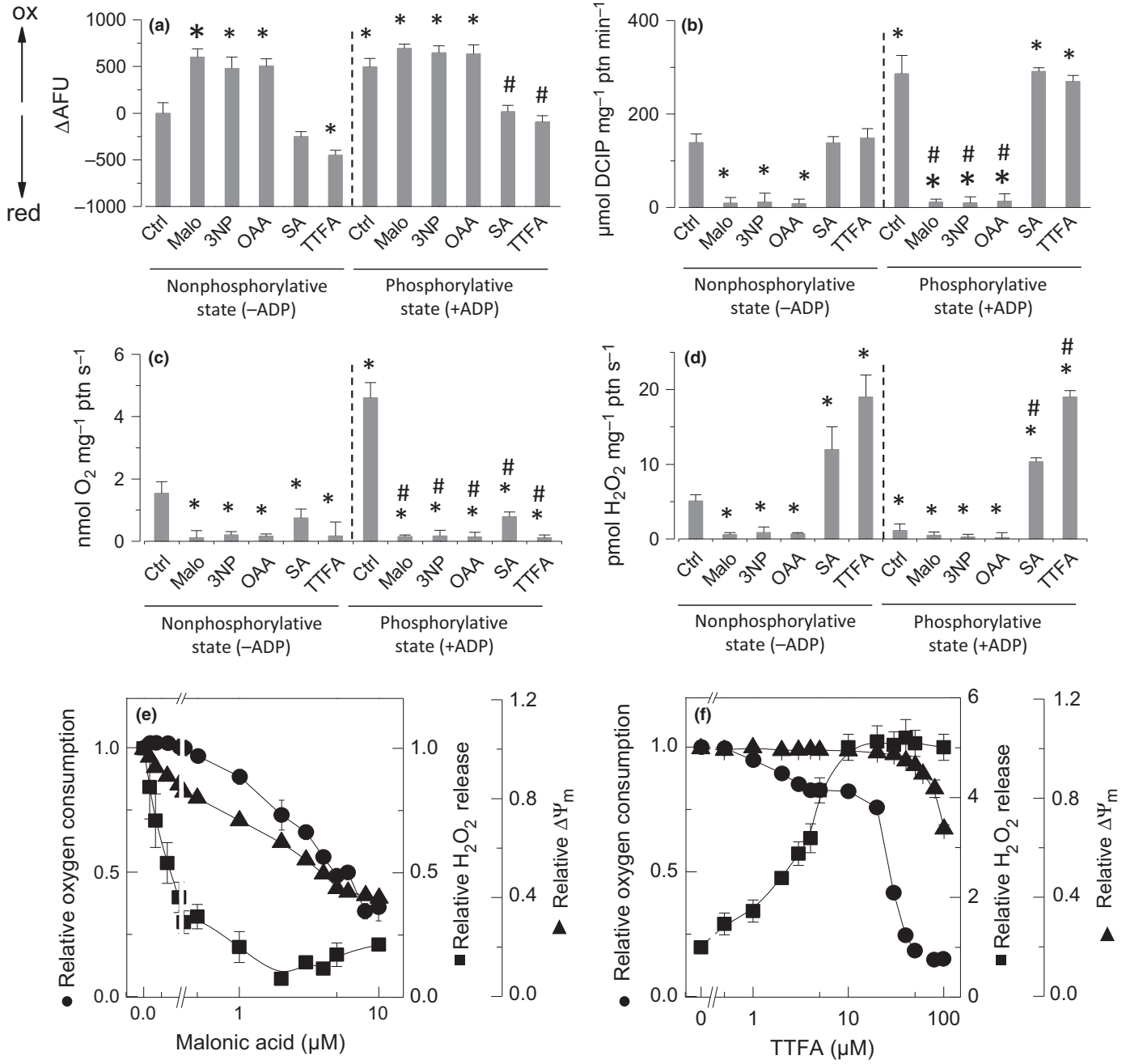

Fig. 2 Effect of competitive and noncompetitive succinate dehydrogenase (SDH) inhibitors on (a) $FADH_2/FAD^+$ redox status, (b) SDH activity, (c) succinate-dependent oxygen consumption, and (d) succinate-dependent reactive oxygen species (ROS) production, in isolated mitochondria. The concentrations of SDH inhibitors were 10 mM malonate (Malo), 1 mM 3-nitropropionic acid (3NP), 1 mM oxaloacetate (OOA), 1 mM salicylic acid (SA) and 100 μM thenoyltrifluoroacetone (TTFA). Succinate-dependent oxygen consumption, ROS production and mitochondrial membrane potential ($\Delta\psi_m$) were evaluated in the presence of increasing concentrations of (e) malonate and (f) TTFA. The reactions were initiated in the presence of 10 mM succinate. The values represent the mean ± SE of eight independent experiments. *, population means are significantly different from the control in the nonphosphorylative state at the 0.05 level; #, population means are significantly different from the control in the phosphorylative state at the 0.05 level. AFU, arbitrary fluorescence unit; Ctrl, control; DCIP, dichlorophenolindophenol; Ox, oxidized; ptn, protein; Red, reduced.

UQ. Nevertheless, the SDH inhibitors produced different effects on succinate-induced ROS production, measured as the release of H_2O_2. The competitive inhibitors, which inhibit succinate oxidation, decreased the ROS production rate, whereas the non-competitive inhibitors increased the ROS production rate (Fig. 2d).

To understand the effect of competitive and noncompetitive SDH inhibition on mitochondrial ROS production, the effect of different concentrations of MA and TTFA, which are specific inhibitors of SDH, on succinate dependent-oxygen consumption, $\Delta\psi_m$ and ROS production was evaluated. Although MA was able to inhibit oxygen consumption as well as membrane

potential and ROS production, the inhibition of oxygen consumption by relatively high concentrations of TTFA was accompanied by a decreased $\Delta\psi_m$ and an increase in the ROS production rate. Interestingly, as demonstrated previously, a low dose of TTFA induced ROS generation without inhibiting oxygen consumption and $\Delta\psi_m$ (Fig. 2e,f).

TTFA induces ROS production by SDH in plant mitochondria

Complex I and complex III are generally considered to be the main sites of mitochondrial ROS production (Kowaltowski *et al.*, 2009; Murphy, 2009; Brand, 2010). However, the TTFA-induced ROS production cannot be assigned to complex I and complex III sites. To determine the site of TTFA-induced ROS production, as well as its specificity, different ETS inhibitors and substrates were used. Mitochondrial ROS production was evaluated using succinate, which is specific to SDH activity, as well as pyruvate and malate, which are substrates linked to complex I activity. In addition to MA and TTFA, the effects of rotenone, a complex I inhibitor, and antimycin A and stigmatellin, specific inhibitors of complex III, were also evaluated. Although antimycin A and stigmatellin are both inhibitors of complex III, antimycin A stabilizes the semiquinone radical in the Q-cycle and increases ROS production by complex III, whereas stigmatellin prevents the semiquinone radical step, thereby impairing complex III-dependent ROS production.

As expected, the addition of antimycin A in the presence of pyruvate and malate, substrates that provide NADH for complex I

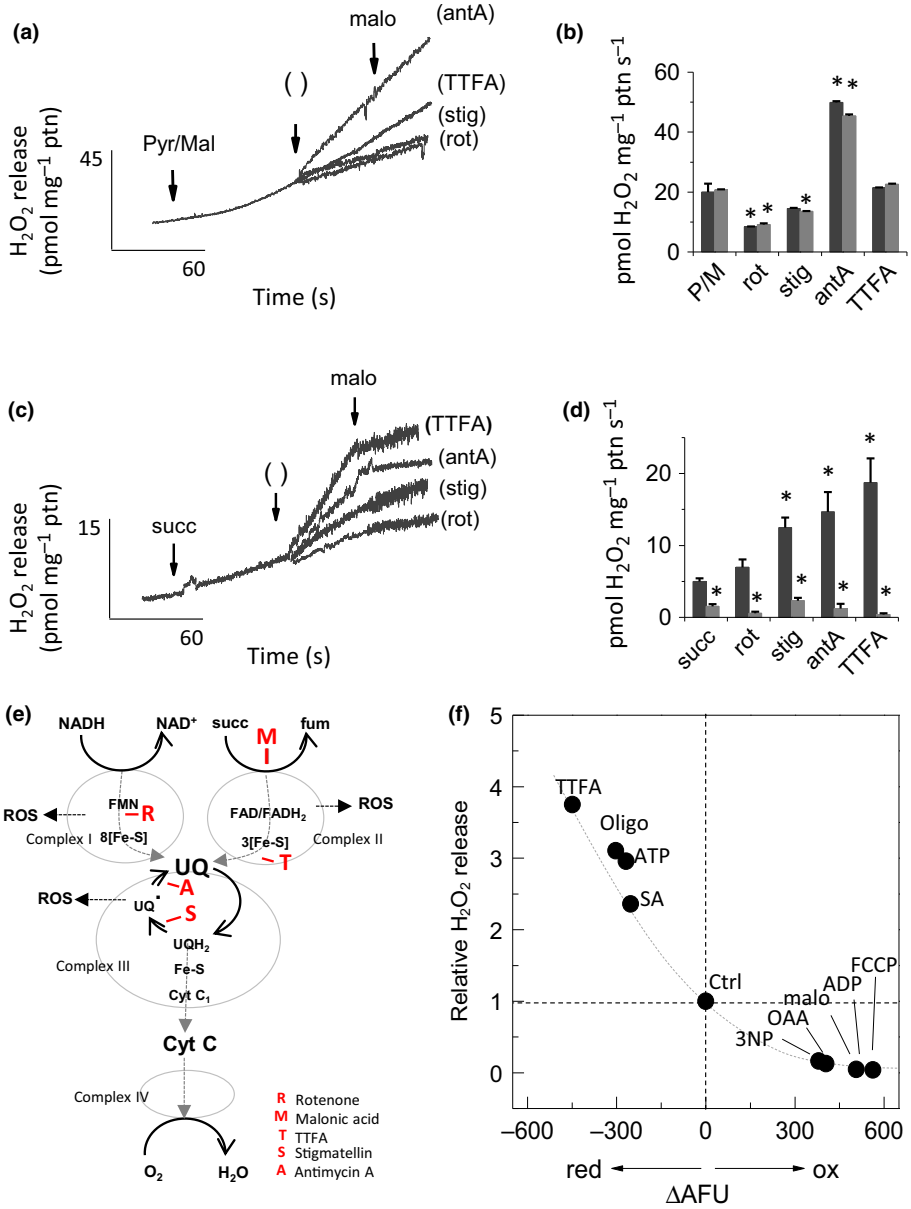

Fig. 3 Mitochondrial oxygen consumption dependent on (a, b) complex I- and (c, d) succinate dehydrogenase (SDH)-linked substrates in *Arabidopsis thaliana* root mitochondria. Dark gray, absence of malonate; light gray, presence of malonate. The reactions were initiated with 10 mM pyruvate/1 mM malate (Pyr/Mal) and succinate (succ). (e) Schematic representation of the electron transport system and the sites of inhibition. (f) The relationship between the SDH redox state and the succinate reactive oxygen species (ROS) production rate in isolated mitochondria. The concentration of inhibitors were 10 mM malonate (malo), 1 mM rotenone (rot), 100 μM thenoyltrifluoroacetone (TTFA), 1 mg ml^{-1} antimycin A (antA), 2 μM stigmatellin (stig), 1 μg ml^{-1} oligomycin (Oligo), 1 mM3-nitropropionic acid (3NP), 1 mM oxaloacetate (OAA) and 1 mM salicylic acid (SA). The carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) and ADP concentrations were 0.5 μM and 1 mM, respectively. The values represent the mean ± SE of eight independent experiments. *, population means are significantly different at the 0.05 level. The empty brackets indicates the point of addition of the inhibitors showed in the right of the graph. AFU, arbitrary fluorescence unit; Cyt C, cytochrome C; FMN, flavin mononucleotide; fum, fumarate; ox, oxidized; Ptn, protein; UQ, ubiquinone; UQH2, ubiquinol.

activity, increased the ROS production rate by *c.* 2.5-fold (Fig. 3a,b). In contrast, the addition of stigmatellin and rotenone decreased the ROS production rate by *c.* 20%. In addition, TTFA or MA did not alter complex I-dependent ROS production. These results confirm that the effects of TTFA and MA on mitochondrial ROS production are specific to SDH activity.

In the presence of succinate as the respiratory substrate, the ROS production rate was also increased *c.* 2.5-fold by antimycin A. However, contrary to what would be expected, the addition of stigmatellin increased the SDH-dependent ROS production rate activity in a similar way to TTFA (Fig. 3c,d). These data demonstrated that TTFA only increases SDH-dependent ROS production, indicating that SDH is a site of ROS production in plant mitochondria. Fig. 3(e) schematically shows the points at which these inhibitors block ETS activity.

The main redox center of SDH is the FAD group, which is bound to the SDH1 subunit, and this prosthetic group is recognized as the site of ROS production in mammalian SDH (Yankovskaya *et al.*, 2003). To determinate the role of the SDH flavin redox state in the ROS production pathways, the relationship between the SDH flavin redox state and SDH-dependent ROS production was analyzed under different conditions. These parameters were evaluated in the presence of competitive SDH inhibitors (3-NP and OAA), noncompetitive SDH inhibitors (SA and TTFA), ATP, which increases the SDH affinity for succinate (Oestreicher *et al.*, 1973), ADP, the substrate of oxidative phosphorylation, oligomycin, an ATP synthase inhibitor, and the H^+ ionophore FCCP, which is able to dissipate the $\Delta\psi_m$. These results demonstrated that the highest rates of ROS production occurred when the SDH flavin was in the reduced state (Fig. 3f).

SDH-dependent ROS production regulates plant growth

To determine the physiological implications of SDH-dependent ROS production, *A. thaliana* germination was evaluated in the presence of TTFA at 10 μM, which induces ROS production without inhibiting SDH activity, and at 100 μM, which both induces ROS production and inhibits SDH activity. Both concentrations were able to impair plant growth (Fig. 4a,b). In addition, the effect of noncompetitive SDH inhibitors on plant growth by hydroponic treatment was also evaluated. In addition to TTFA, which is a specific SDH inhibitor, the effect of nonspecific SDH inhibitors such as SA (Norman *et al.*, 2004) and nitric oxide (NO) (Simonin & Galina, 2013), which perform other biological roles but are also noncompetitive SDH inhibitors, was also examined. Both concentrations of TTFA inhibited shoot and root growth by approximately five-fold, indicating that the inhibitory effect of TTFA on plant growth is not solely dependent on SDH activity inhibition. *S*-Nitroso-*N*-acetylpenicillamine (SNAP), an NO donor, did not inhibit shoot growth, whereas 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), an NO scavenger, increased shoot growth by 20%. SNAP inhibited root growth by 70%, and this effect was not fully reversed by CPTIO. In addition, SA inhibited shoot and root growth approximately nine- and four-fold, respectively (Fig. 4c,d). Similar effects were also observed in rice plants (Fig. S2).

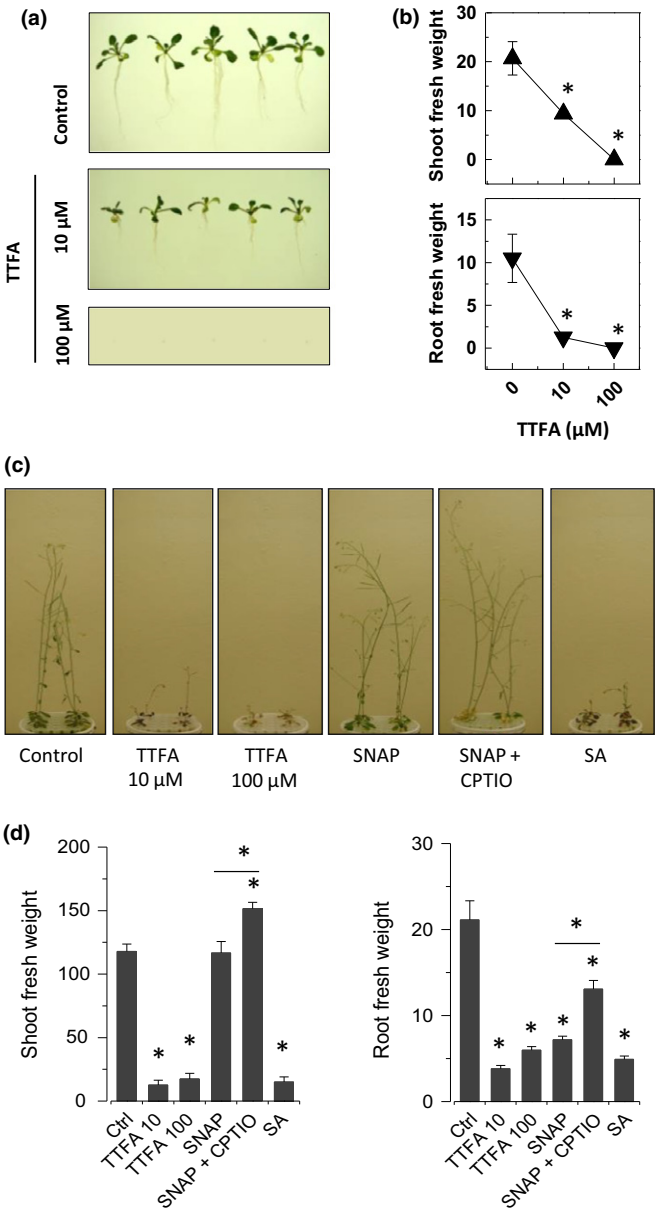

Fig. 4 (a, b) Effect of thenoyltrifluoroacetone (TTFA) on *Arabidopsis thaliana* germination demonstrated in 5-d-old plants. (c, d) In addition, the effect of TTFA and other noncompetitive succinate dehydrogenase (SDH) inhibitors on *A. thaliana* growth was evaluated in 1-month-old plants. The concentrations of the SDH inhibitors were 10 and 100 μM TTFA, 100 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 1 mM salicylic acid (SA). The 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) concentration was 200 μM. The values represent the mean ± SE of three independent experiments with 10 individuals. *, population means are significantly different at the 0.05 level. Ctrl, control.

To determine whether the decreased plant growth was attributable only to the inhibition of electron flow through the ETS, we also evaluated the effect of competitive SDH inhibition on plant growth. Curiously, low doses of MA, a competitive inhibitor of SDH, increased shoot and root growth (Fig. 5a,b), and this effect was accompanied by decreased H_2O_2 release in roots (Fig. 5c). However, high concentrations of MA inhibited oxygen consumption in roots, resulting in reduced plant growth, along

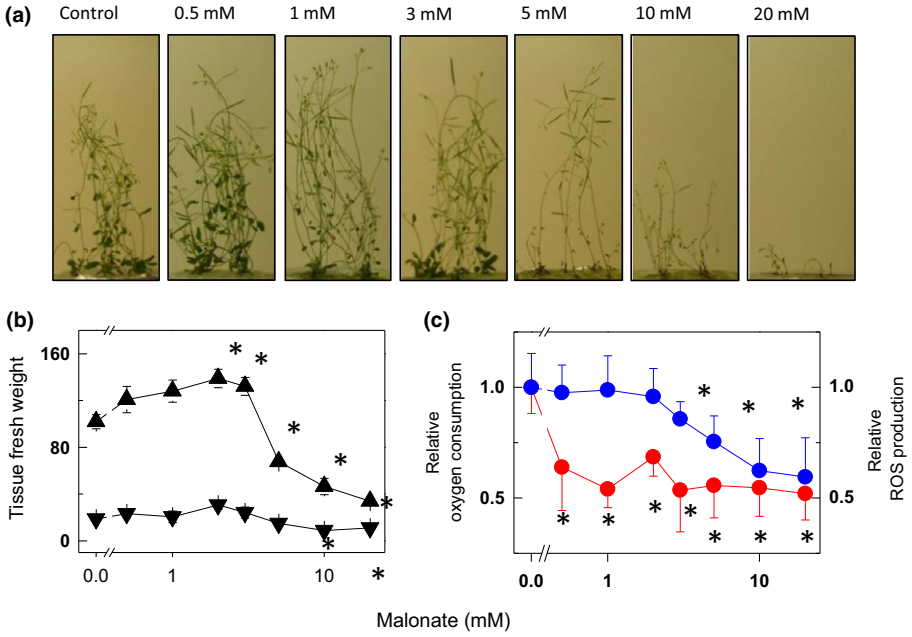

Fig. 5 (a, b) Effect of increasing malonate concentrations on *Arabidopsis thaliana* growth. (c) Effect of malonate on hydrogen peroxide (H_2O_2) release (red) and oxygen consumption (blue) in *A. thaliana* roots. One-month-old plants were used, and the values represent the mean ± SE of three independent experiments with 10 individuals. *, population means are significantly different at the 0.05 level.

with decreased ROS release. A similar result was also previously demonstrated in mutant plants, which showed lower SDH activity accompanied by decreased ROS production and increased growth (Araújo *et al.*, 2011; Fuentes *et al.*, 2011; Gleason *et al.*, 2011).

In rice, low doses of MA were not able to stimulate plant growth or decrease H_2O_2 release, but, as in *A. thaliana*, high MA doses led to reduced oxygen consumption and, consequently, impaired plant growth (Fig. S3). These results demonstrate that the effect of SDH inhibition on plant growth is dependent on the mechanism of SDH inhibition, which modulates mitochondrial ROS production.

ROS production derived from SDH regulates the expression of genes related to plant development and stress responses

To understand the effect of SDH-dependent ROS production on plant growth, the expression of genes related to plant development, including cyclin (*CYC*) genes, cyclin-dependent kinase (*CDK*) genes and histone H4 genes, was verified by RT-qPCR in plants treated with the specific competitive and noncompetitive SDH inhibitors MA and TTFA, respectively. Figure 6(a,b) presents the treatment schemes. For these treatments, 10 μM TTFA was used because, at this concentration, TTFA increases SDH-dependent ROS production without inhibiting SDH activity. TTFA treatment reduced the expression of *AtCYCA3*, *AtCYC3;1*, *AtCDKB2;1* and *AtH4* by approximately three-fold, and this effect was similar to that of direct treatment with H_2O_2 (Fig. 6c). However, pretreatment with MA prevented the inhibitory effect of TTFA on gene expression (Fig. 7d), confirming the requirement for SDH modulation. A similar effect on rice gene expression was also observed (Fig. S4).

Previous studies have demonstrated that the *A. thaliana SDH1-1/sdh1-1* heterozygous and *dsr1* mutants showed reduced SDH activity and, consequently, low mitochondrial ROS production, as well as increased plant growth (Fuentes *et al.*, 2011; Gleason *et al.*, 2011). To verify the effect of SDH activity on the regulation of gene expression, an *in silico* analysis was performed to compare the transcriptional profiles of wild-type (WT) and *dsr1* mutant plants. Among the 22746 genes evaluated, 1798 were up-regulated and 65 were down-regulated in *dsr1* compared with WT (Fig. 6e). Ontological analyses showed that, among the up-regulated genes, those related to biosynthetic processes and nitrogen metabolism were well represented (17% and 14%, respectively). Finally, these results demonstrate that the modulation of SDH activity by mutations or pharmacological inhibitors produces similar effects on the ROS production pathways and, consequently, on the modulation of gene expression.

Gleason *et al.* (2011) demonstrated that *dsr1* mutant *A. thaliana* plants have impaired biotic stress responses. To verify the importance of SDH activity in plant stress responses, the transcription profile of an association network of antioxidant proteins in response to SA was analyzed by comparing WT and *dsr1* plants *in silico*. This network was created using the STRING database (Snel *et al.*, 2000) with genes related to antioxidant metabolism in *A. thaliana*. In WT *A. thaliana*, SA treatment induced the antioxidant response, up-regulating the expression of genes in the association network, compared with plants in the absence of SA. However, in *dsr1* mutant plants, whose SDH activity and ROS concentrations are reduced, this response to SA treatment was impaired (Fig. 7a).

In addition, the relative expression of the genes encoding these antioxidant enzymes was analyzed in response to SA. In WT plants, the up-regulated genes were mainly members of the glutathione-S-transferase (GST) family, but the expression of other genes encoding antioxidant enzymes was also increased in response to stress. In *dsr1* mutants, the antioxidant response is prevented. In addition, the expression of some GST enzymes also decreased (Fig. 7b).

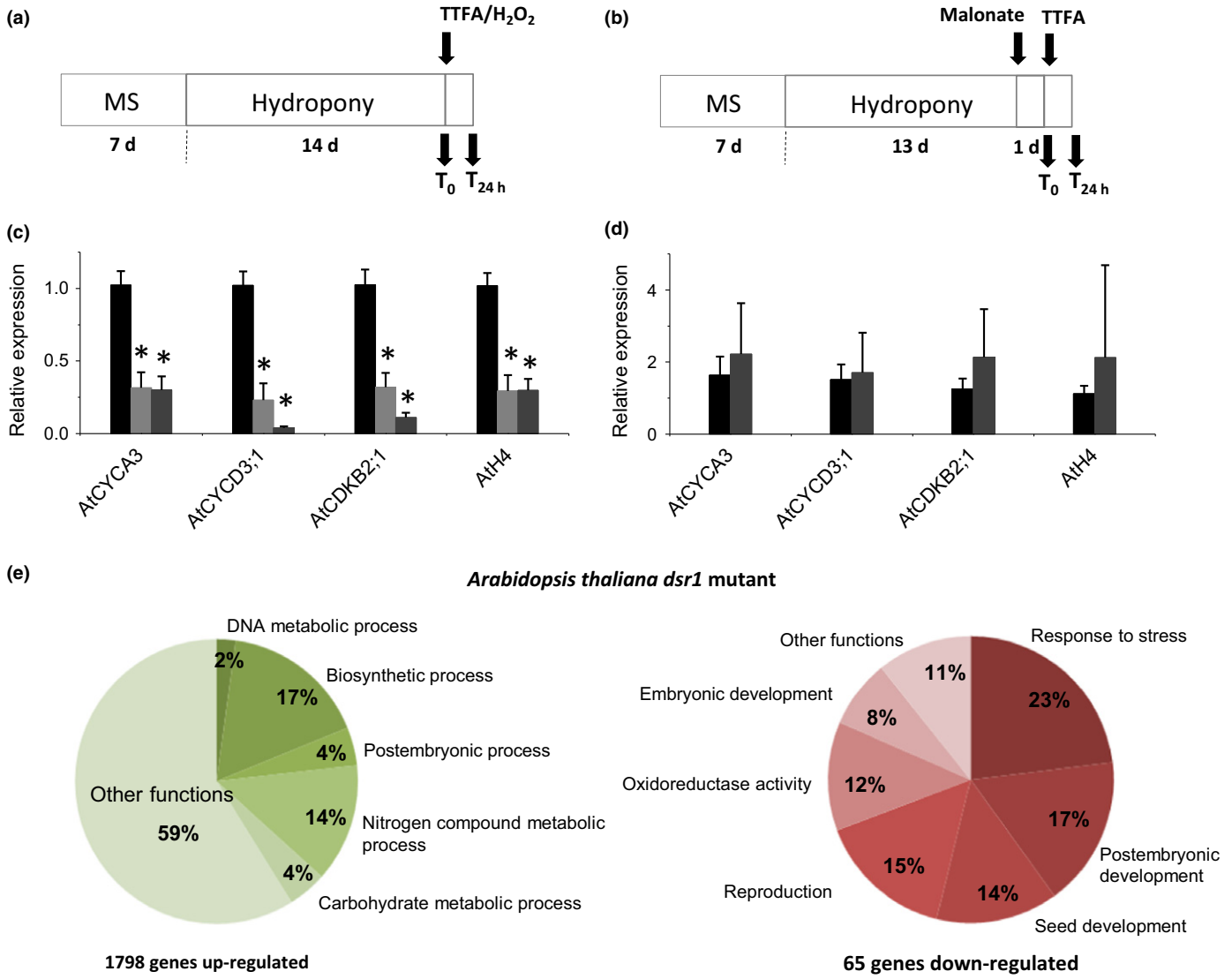

Fig. 6 Quantitative real time-PCR evaluation of the effect of succinate dehydrogenase (SDH) inhibition on developmental gene expression in *Arabidopsis thaliana*. (a, b) Schematic representation of plant treatments. MS, Murashige & Skoog medium. (c) Relative expression of *CYCLIN A3* (*AtCYCA3*), *AtCYCD3;1*, *CYCLIN-DEPENDENT KINASE B2;1* (*AtCDKB2;1*), and histone H4 from *Arabidopsis thaliana* (*AtH4*) in plants treated with hydrogen peroxide (H_2O_2) or 10 μM thenoyltrifluoroacetone (TTFA). Black, control; light gray, H_2O_2; dark gray, TTFA. (d) Relative expression of *AtCYCA3*, *AtCYCD3;1*, *AtCDKB2;1*, and *AtH4* in plants pretreated with 10 mM malonate before TTFA treatment. Black, control; dark gray, TTFA. The values represent the mean ± SE of three independent experiments. *, population means are significantly different at the 0.05 level. (e) The ontological analysis of differentially expressed genes in the *disrupted stress response 1* (*dsr1*) mutant of *A. thaliana*.

To confirm that the modification of antioxidant response-related gene expression observed in mutant plants was attributable to decreased SDH-dependent ROS production and not just to decreased SDH activity, the expression of GST enzymes in response to 10 μM TTFA treatment was experimentally evaluated by RT-qPCR. As with H_2O_2 treatment, TTFA was able to increase *AtGSTF8* expression (Fig. 8a,b). The effect of other non-competitive SDH inhibitors, such as SA and NO, was also evaluated. Both inhibitors induced GST expression, and this effect was impaired by MA pretreatment (Fig. 8c,d), demonstrating the specificity of SDH modulation in this response. In addition, the NO effect was also impaired by CPTIO, an NO scavenger (Fig. 8d). The expression of GST phi classes 5 and 10 (*OsGSTF5* and *OsGSTF10*), which are responsive to oxidative stress (Jain *et al.*, 2010; Gleason *et al.*, 2011), was also evaluated in rice. The effects of competitive and noncompetitive inhibitors on gene expression mimicked those observed in *A. thaliana* (Fig. S5).

Discussion

ETS inhibitors are useful tools for determining different aspects of mitochondrial function, including oxygen consumption, $\Delta\psi_m$ and ROS production. SDH inhibitors can be

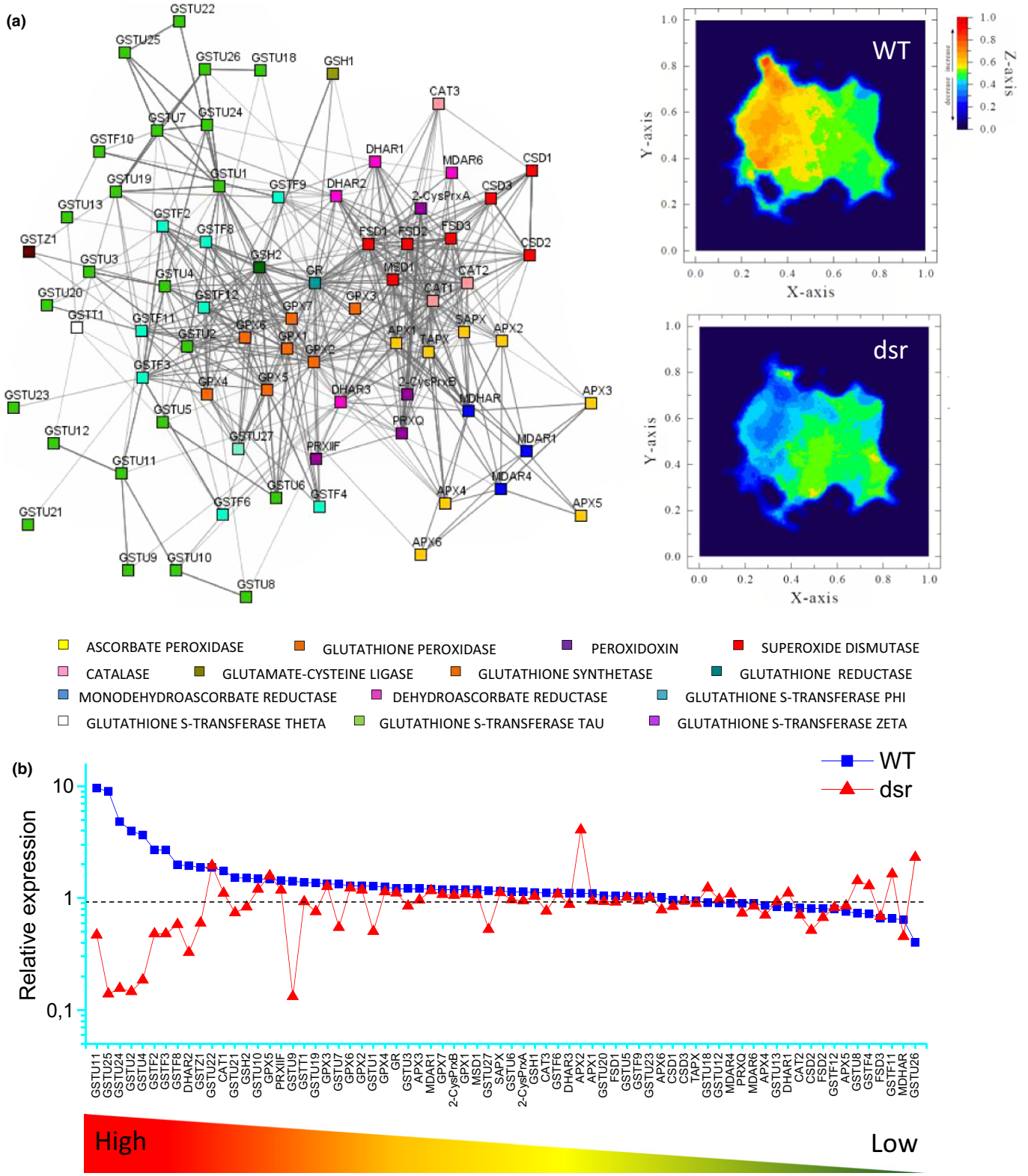

Fig. 7 Effect of salicylic acid treatment on *Arabidopsis thaliana* antioxidant gene network expression. (a) Heatmap demonstrating the antioxidant gene network expression in wild type (WT) and *disrupted stress response 1* (*dsr1*) mutants treated with salicylic acid. (b) Relative expression of antioxidant genes in WT and *dsr1* mutants treated with salicylic acid.

divided into two subgroups: competitive inhibitors that bind to the succinate-binding site, such as MA, 3NP (Alston *et al.*, 1977) and other TCA cycle metabolites, including malate, fumarate, citrate and especially OAA (Gutman *et al.*, 1971; Kearney *et al.*, 1972); and noncompetitive inhibitors that bind to the Q-site, including TTFA (Mowery *et al.*, 1976),

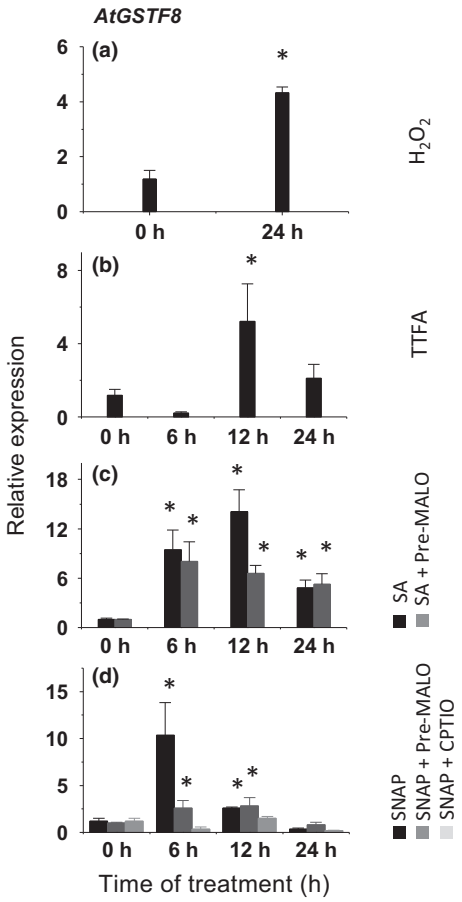

Fig. 8 Evaluation of *GLUTATHIONE-S-TRANSFERASE PHI TYPE 8* (*AtGSTF8*) expression in *Arabidopsis thaliana* treated with (a) hydrogen peroxide (H_2O_2), (b) 10 μM thenoyltrifluoroacetone (TTFA), (c) 1 mM salicylic acid (SA), or (d) 100 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP). The concentrations of malonate (MALO) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) were 10 mM and 200 μM, respectively. The values represent the mean ± SE of three independent experiments. *, population means are significantly different at the 0.05 level.

ATPenins (Miyadera *et al.*, 2003), vitamin E analogs (Dong *et al.*, 2008, 2011a,b), SA (Norman *et al.*, 2004) and NO (Simonin & Galina, 2013).

Previous studies demonstrated that mammalian SDH can be a major source of mitochondrial ROS production when SDH activity is blocked by a noncompetitive inhibitor (Quinlan *et al.*, 2012), and Chen *et al.* (2007) demonstrated that TTFA, a noncompetitive and specific SDH inhibitor, is able to induce ROS production.

In the present study, we have evaluated the capacity of SDH to generate mitochondrial ROS and thus regulate development and stress-related gene expression in plants. Our results demonstrated that SDH is a direct source of ROS in plant mitochondria and that the induction of ROS production by specific SDH inhibitors impairs plant growth. In isolated mitochondria, the competitive SDH inhibitors MA, 3NP and OAA increased the oxidative state of SDH (Fig. 2a) through the inhibition of succinate oxidation (Fig. 2b). This effect was accompanied by decreased mitochondrial H_2O_2 production (Fig. 2c). However, the noncompetitive inhibitors SA and TTFA did not inhibit succinate oxidation (Fig. 2b), increasing the reduced state of SDH (Fig. 2a) and mitochondrial H_2O_2 production (Fig. 2c). In addition, the effect of MA and TTFA on mitochondrial ROS production is specific to SDH modulation and does not depend on other ETS complexes such as complex I and complex III, and the mitochondrial ROS production is proportional to the reduced state of SDH (Fig. 3). These data confirm the central role of SDH in mitochondrial ROS production because, in addition to being essential for the generation of ROS by complex I and complex III (Kowaltowski *et al.*, 2009; Murphy, 2009; Brand, 2010; Møller & Sweetlove, 2010), SDH is a direct source of ROS generation in plant mitochondria. Similar results were previously obtained in mammalian mitochondria, where ATPenins, a noncompetitive inhibitor, increased mitochondrial ROS production (Quinlan *et al.*, 2012), demonstrating that SDH is an important site of ROS production in both animals and plants.

As a consequence of the importance of SDH in mitochondrial energy generation and in ROS production, SDH activity needs to be finely regulated. Endogenous molecules such as SA or NO, which are produced mainly under stress conditions (Raskin, 1992; Delledonne *et al.*, 1998) and perform other signaling roles in plant development and tolerance, are able to regulate SDH activity and induce ROS production (Fig. 2d). Noncompetitive SDH inhibition induced oxidative stress and impaired plant growth (Fig. 4), revealing the influence of SDH-dependent ROS production in plant development. Interestingly, a low TTFA concentration (10 μM) increased mitochondrial ROS production, limiting plant growth without inhibiting SDH activity and, consequently, mitochondrial respiration (Figs 1c,d, 2f). These results demonstrate that the decrease in plant growth was not attributable only to SDH inhibition but was also attributable to the induction of SDH-dependent ROS production by noncompetitive inhibition.

In contrast, competitive SDH inhibition prevented succinate oxidation and, consequently, mitochondrial ROS production (Fig. 2a–d). Thus, a low MA concentration, which did not completely inhibit SDH activity (Fig. 2e), induced plant growth (Fig. 5a). As demonstrated previously in mutant plants (Araújo *et al.*, 2011; Fuentes *et al.*, 2011; Gleason *et al.*, 2011), this effect was accompanied by a decrease in H_2O_2 release from plant tissues without any inhibition of mitochondrial respiration (Fig. 5c).

SDH-dependent ROS production impaired the expression of different genes related to the cell cycle, suggesting a possible mechanism by which ROS induced by SDH/complex II limits plant growth. In *A. thaliana*, TTFA treatment decreased the expression of *AtCYCA3*, *AtCYC3;1*, *AtCDKB2;1* and *AtH4* (Fig. 6c), and this effect was impaired by MA pretreatment (Fig. 6d). A similar result was also observed in rice, in which MA pretreatment induced the expression of CYC and CDK and prevented the TTFA effect (Fig. S4). These data were confirmed by ontological analysis of the transcriptional profiles of WT and the *dsr1* mutant, which shows reduced SDH activity and ROS production, which indicated that the lack of SDH activity induces

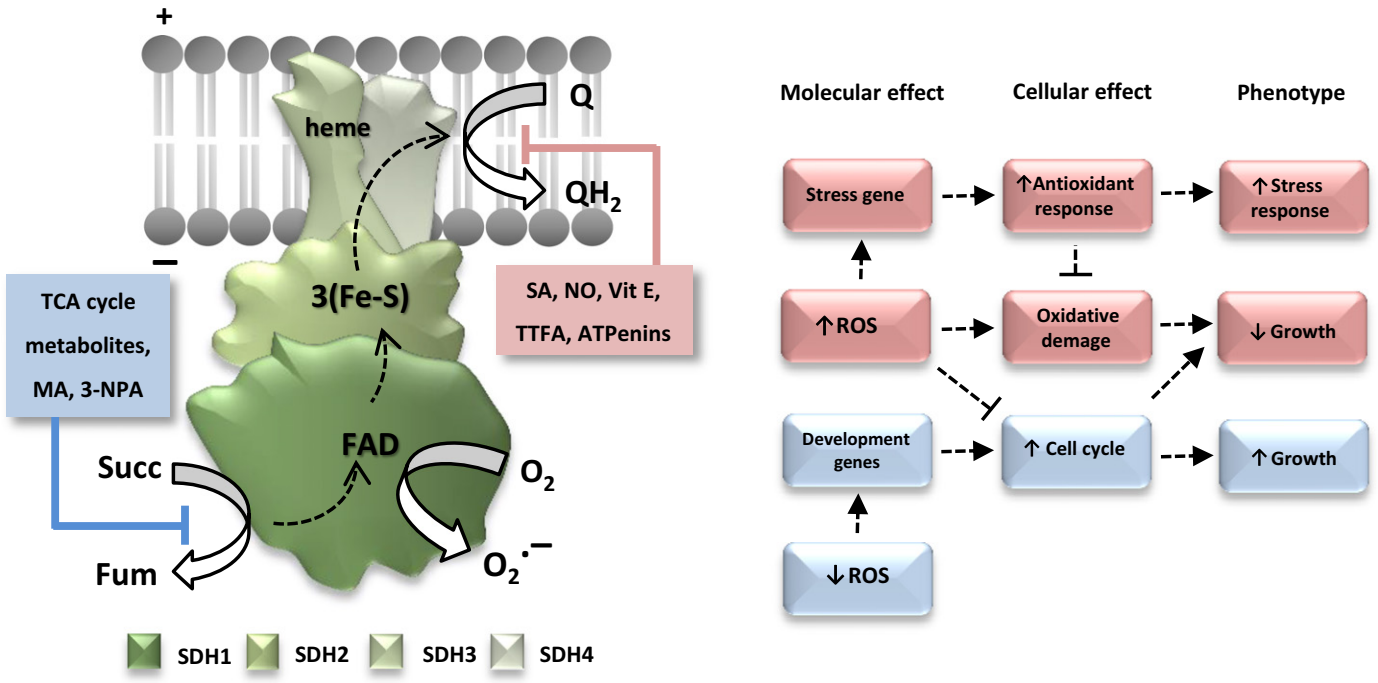

Fig. 9 Effects and phenotypes of succinate dehydrogenase (SDH) inhibition in plants. Noncompetitive inhibitors (pink) such as salicylic acid (SA), nitric oxide (NO), vitamin E, thenoyltrifluoroacetone (TTFA) and ATPenins increase SDH-dependent reactive oxygen species (ROS) production. The increased ROS production leads to oxidative damage and impairs the cell cycle, resulting in decreased growth. However, SDH-dependent ROS production can also signal the antioxidant responses, increasing plant stress responses. In contrast, competitive inhibitors (blue) such as tricarboxylic acid (TCA) cycle metabolites (oxaloacetate, fumaric acid, citric acid and malate), malonate (MA), and 3-nitropropionic acid (3-NP) decrease succinate oxidation and, consequently, SDH-dependent ROS production. This effect decreases plant stress responses but enables the expression of genes related to development, as verified in *disrupted stress response 2* (*dsr1*) mutants. It is noteworthy that full SDH inhibition decreases mitochondrial function, leading to decreased ATP production and the perturbation of organic acids, making unviable gene knockout (KO) plants for SDH. fum, fumarate; Q, ubiquinone; QH2, ubiquinone reduced/ubiquinol; succ, succinate.

the expression of genes specifically related to biosynthetic processes and nitrogen metabolism (Fig. 6e). Finally, these results indicate that SDH can be a limiting factor in plant growth through mitochondrial ROS generation.

Among the genes that were down-regulated in the *dsr1* mutant, a large number were involved in stress responses (Fig. 6e). Furthermore, these plants had an impaired stress response and were more susceptible to fungal and virulent bacterial pathogens (Gleason *et al.*, 2011), indicating the importance of SDH-derived ROS in the expression of plant defense genes. This result was confirmed by analyzing an association network of antioxidant genes in response to SA-induced stress. In WT plants, SA induced the expression of various genes, mainly including GST family members. However, the response to SA was impaired in the *dsr1* mutant (Fig. 7). The importance of SDH-dependent ROS production in stress responses was confirmed by RT-qPCR. The induction of ROS production by noncompetitive inhibitors such as TTFA, SA and NO increased the expression of *AtGSTF8*, and MA pretreatment, which abolished the SDH-dependent ROS production, impaired this effect (Fig. 8), as verified previously in *dsr1* mutants. In rice, the expression of *OsGSTF5* and *AtGSTF10* was also induced by H_2O_2, and noncompetitive SDH inhibitors induced gene expression in an MA-sensitive way (Fig. S5), confirming the importance of SDH activity in this pathway.

In conclusion, SDH is an important site of ROS production in plant mitochondria, in addition to complex I and complex III, which are well known as sites of ROS production. Different molecules can physiologically control SDH-dependent ROS production by modulating SDH activity. Noncompetitive SDH inhibitors, including vitamin E analogs (Dong *et al.*, 2008, 2011a,b), SA (Norman *et al.*, 2004) and NO (Simonin & Galina, 2013), inhibit UQ reduction, thereby increasing the reduced state of SDH and enhancing H_2O_2 release (Fig. 9, pink). This effect impairs the expression of genes related to plant development and limits plant growth. In addition, ROS delivered from SDH can activate the expression of stress-related genes, thereby inducing antioxidant responses and stress tolerance (Fig. 9, pink). Previous studies have demonstrated that SA is a key component in plant resistance, and its production is induced during and following plant stress (McCue *et al.*, 2000; Lewsey *et al.*, 2009). In addition, a deficiency in SA signaling impairs defense responses and increases susceptibility to pathogen attack (Takahashi *et al.*, 2004; Sanchéz *et al.*, 2010; Jovel *et al.*, 2011). NO production is also induced by plant stress, and NO participates in the activation of pathogenesis-related pathways (Delledonne *et al.*, 1998). Thus, we propose that SDH-dependent ROS represents an additional mechanism to explain plant resistance induced by SA or NO. In contrast, competitive SDH inhibitors such as TCA cycle metabolites (Gutman *et al.*, 1971; Kearney *et al.*, 1972), which

are considered biosynthetic intermediates, inhibit succinate oxidation and SDH-dependent ROS production, leading to an increase in the expression of genes related to plant development and growth (Fig. 9, blue). Therefore, we suggest that SDH is a site of ROS generation in plant mitochondria and that SDH plays an important role in regulating plant development and responses to stress.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The noncompetitive inhibition of SDH induces ROS generation in rice tissues.

Fig. S2 The noncompetitive inhibitors of SDH impair germination, and shoot and root growth in rice.

Fig. S3 Low doses of a competitive inhibitor of SDH decrease ROS production in rice tissue and have no effect on plant growth.

Fig. S4 TTFA treatment impairs developmental gene expression in rice.

Fig. S5 The noncompetitive inhibitors of SDH induce *OsGSTF5* and *OsGSTF10* expression in rice.

Table S1 Sequences of primers used in quantitative real-time PCR experiments

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