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CATALASE2 Coordinates SA-Mediated Repression of Both Auxin Accumulation and JA Biosynthesis in Plant Defenses

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SUMMARY

Plants defend against pathogen attack by modulating auxin signaling and activating the salicylic acid (SA) and jasmonic acid (JA) signaling pathways. SA and JA act antagonistically in resistance to specific pathogen types, yet how plants coordinate these phytohormones remains elusive. Here we report that biotrophic-pathogen-induced SA accumulation dampens both auxin and JA synthesis by inhibiting CATALASE2 (CAT2) activity in the model plant *Arabidopsis*. SA suppression of CAT2 results in increased H_2O_2 levels and subsequent sulfenylation of tryptophan synthetase β subunit 1, thus depleting the auxin biosynthetic precursor tryptophan. In addition, we find that CAT2 promotes JA biosynthesis by facilitating direct interaction of the JA biosynthetic enzymes ACX2 and ACX3, and thus SA repression of CAT2 inhibits JA accumulation. As such, the *cat2-1* mutant exhibits increased resistance to biotrophic pathogens and increased susceptibility to necrotrophic pathogens. Our study illustrates how CAT2 coordinates SA repression of auxin accumulation and JA biosynthesis in plant defense.

INTRODUCTION

Plants have evolved sophisticated mechanisms for defense against necrotrophic and biotrophic pathogens (Zheng et al., 2012). Successful defense against these pathogens depends on precise, complex regulation of phytohormones, including salicylic acid (SA) and jasmonic acid (JA). In general, plants activate SA-mediated defense against biotrophic pathogens and JA-induced defense against herbivorous insects or necrotrophic pathogens, with a few exceptions (Spoel and Dong, 2008).

The SA defense pathway plays an essential role in plant defense against biotrophic pathogens such as *Pseudomonas syringae pv. tomato* DC3000 (*Pst* DC3000). Infection by *Pst* DC3000 induces plants to produce and accumulate SA, which promotes the accumulation of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) in the nucleus (Spoel and Dong, 2008). Nuclear NPR1 interacts with TGA transcription factors and modulates the expression of defense-related genes, including pathogenesis-related (*PR*) genes.

The JA defense pathway plays an essential role in plant defense against herbivores and necrotrophic pathogens such as *Botrytis cinerea* (*B. cinerea*). *B. cinerea* infection activates JA biosynthesis and signaling pathways to promote the immune response (Zheng et al., 2012). In JA biosynthesis, the precursors 3-oxo-2-(2′-[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) and 3-oxo-2-(2′-[Z]-pentenyl)-cyclopentane-1-hexanoic acid (OPC-6) are metabolized to JA by several rounds of β-oxidation catalyzed with three core enzymes—acyl-CoA oxidase (ACX), multifunctional protein, and 3-ketoacyl-CoA-thiolase—in the peroxisome. The JA receptor CORONATINE INSENSITIVE1 (COI1) functions as an F-box protein of the Skp/Cullin/F-box complex (SCF^{COI1}) for ubiquitin-mediated degradation of JAZ proteins, releasing the repression of downstream transcription factors such as MYC2, ERF1, and ORA59. These transcription factors then activate JA-responsive genes like *PDF1.2* against necrotrophs and *VSP2* against herbivorous insects or wounding (Wasternack and Hause, 2013)

JA and SA act antagonistically to mediate defense responses: JA can repress SA-mediated defense during plant-pathogen or plant-herbivore interactions. For instance, treatment with coronatine (COR), a JA analog that can activate JA signaling, represses SA and promotes bacterial growth in plants (de Torres Zabala et al., 2009). Consistent with this, a COR-deficient *Pst* DC3000 mutant exhibits reduced virulence in *Arabidopsis* due to increased SA (Brooks et al., 2005). Also, the COR-insensitive *Arabidopsis* mutants *coi1* and *myc2/jin1* show enhanced resistance to *Pst* DC3000 (Laurie-Berry et al., 2006). A recent report indicates that COR represses SA accumulation by regulating the expression of genes involved in SA biosynthesis and metabolism (Zheng et al., 2012). Similarly, more than two decades ago, Peña-Cortés et al. reported that SA and SA derivatives repress the expression of JA-biosynthesis or JA-responsive genes (Peña-Cortés et al., 1993). Later, two reports further indicated that SA also affects the expression of JA-biosynthesis genes *lipoxygenase2* and *allene oxide synthase* (Laudert and Weiler 1998; Spoel et al., 2003). In addition, *Arabidopsis* NahG plants, which cannot accumulate SA, accumulate higher levels of JA and show enhanced expression of JA-responsive genes

when infected by *Pst* DC3000, indicating that SA suppresses both JA metabolism and signaling (Spoel et al., 2003). Several components of SA signaling also act in suppression of JA signaling, including NPR1, the lipase-like proteins ENHANCED DISEASE SUSCEPTIBILITY1 and PHYTOALEXIN-DEFICIENT4, fatty acid desaturase SUPPRESSOR OF SA INSENSITIVITY2, glutaredoxin GRX480, and class II TGA and WRKY transcription factors (Spoel et al., 2003; Van der Does et al., 2013). Until recently, SA was thought to strongly reduce the accumulation of ORA59, a JA-responsive transcription factor, causing inhibition of the expression of JA-responsive genes (Van der Does et al., 2013). However, how SA modulates JA metabolism remains unknown.

The phytohormone auxin also participates in plant defenses against biotrophs (Spoel and Dong, 2008). For example, exogenous auxin loosens plant cell walls and promotes cell elongation, accelerating the development of *Pst* DC3000 symptoms, since loosening of the plant cell wall facilitates bacterial entry into the apoplast. Pathogens such as *Agrobacterium tumefaciens*, *Hyaloperonospora parasitica* Noco2, and *Pst* DC3000 produce auxin or auxin analogs and thus enhance the susceptibility of plants, indicating negative roles of auxin during plant defense responses to biotrophic pathogens (Kazan and Manners, 2009). Plants can repress auxin signaling for defense against pathogen invasion. The plant receptor FLS2 recognizes the bacterial peptide flg22 to induce miR393 for degradation of transcripts of the auxin-receptor genes *TIR1*, *AFB2*, and *AFB3*, leading to enhanced resistance to *Pst* DC3000 (Navarro et al., 2006). SA also causes global repression of auxin-related genes, including *TIR1*, resulting in stabilization of the Aux/indole-3-acetic acid (IAA) repressor to inhibit auxin responses (Wang et al., 2007). In addition, mutants over-accumulating SA have lower IAA levels than wild-type for increased resistance to pathogens, suggesting that the inhibitory effect on auxin is part of SA-mediated disease-resistance mechanisms (Wang et al., 2007). However, how SA reduces auxin accumulation during the plant response to pathogens remains elusive.

Reactive oxygen species (ROS) also play a central role in plant defense against pathogen infection (Zhou et al., 2015). The mutant *rbohF*, which has decreased NADPH oxidase activity, has fewer ROS and thus has reduced resistance to bacterial infection (Torres et al., 2013). Consistent with this, exogenous H_2O_2 enhances plant pathogen resistance (Hong et al., 2013), and the H_2O_2-scavenging enzyme catalase (CAT) also participates in plant disease resistance. Tobacco lines with reduced catalase activity are more resistant to bacterial pathogens, whereas lines overexpressing *CAT1* are less resistant to these pathogens (Mittler et al., 1999). Similarly, in *Arabidopsis*, the *cat2-1* mutation confers enhanced resistance to *Pst* DC3000 (Chaouch et al., 2010). However, how H_2O_2 mediates plant immune responses remains largely unknown.

H_2O_2 mediates protein sulfenylation, which plays important roles in various cellular processes in mammals and microbes (Karisch et al., 2011; Kim et al., 2002). H_2O_2 can modify the thiols (−SH) of cysteine residues to sulfenic acid (−SOH), which can be reversed to −SH by reductants. Sulfenic acid can be irreversibly oxidized to sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$) (Saurin et al., 2004). In mammals, the sulfenylation of the ion channel protein KV1.5 in human heart regulates its stability and diverts channel from a recycling pathway to degradation under oxidative stress (Svoboda et al., 2012). In yeast, H_2O_2 sulfenylates YAP1 and activates its transcriptional activity (Okazaki et al., 2007). Similarly, in *E. coli*, H_2O_2 regulates gene expression by sulfenylating the transcription factor OxyR (Zheng et al., 1998). However, H_2O_2-mediated protein sulfenylation has not been functionally analyzed in plants, although a recent paper shows that plant proteins can be also sulfenylated (Waszczak et al., 2014).

Here, we describe how plants coordinate SA-mediated repression of auxin accumulation and JA biosynthesis upon *Pst* DC3000 infection. *Pst* DC3000-mediated accumulation of SA decreases auxin levels by inhibiting CAT2 activity, which reduces the accumulation of the auxin precursor tryptophan through H_2O_2-mediated sulfenylation of tryptophan synthetase β subunit 1 (TSB1). This also results in low JA levels, as SA suppresses CAT2 stimulation of ACX2 and ACX3 activity in JA biosynthesis.

RESULTS

Catalase Acts in SA-Mediated Resistance to *Pst* DC3000 by Reducing IAA Accumulation

The *cat2-1* mutant has enhanced resistance to *Pst* DC3000 (Chaouch et al., 2010). We previously found that *cat2-1* accumulates less IAA than the wild-type and that changes of IAA levels in *cat2-1* require accumulation of H_2O_2 (Gao et al., 2014). Here we examined whether the enhanced resistance of *cat2-1* to *Pst* DC3000 was due to decreased IAA levels. Upon *Pst* DC3000 infection, *cat2-1* displayed higher resistance than the wild-type, as reported by Chaouch et al. (2010) (Figure 1A), but displayed decreased auxin signaling based on GUS staining of *cat2-1 DR5::GUS* plants (Figure 1B). This reduced auxin signaling could be due to decreased IAA accumulation; indeed, the IAA level was much lower in *cat2-1* than in the wild-type when challenged with *Pst* DC3000 (Figure 1C). These data suggest that the increased disease resistance could result from decreased IAA in *cat2-1* plants. This conclusion was further supported by using *cat2-1 CAT2::iaaM-11*, which accumulates more IAA (Gao et al., 2014). Compared with *cat2-1*, the *cat2-1 CAT2::iaaM-11* plants were more susceptible to *Pst* DC3000 infection (Figure 1A).

Plants infected with biotrophic pathogens such as *Pst* DC3000 activate SA biosynthesis and signaling. To examine whether changes in IAA levels were involved in SA-mediated pathogen resistance in *cat2-1*, we crossed *cat2-1* to *sid2-2*, in which mutation in the SA biosynthetic gene *ISOCHORISMATE SYNTHASE1 (ICS1)* suppresses SA biosynthesis. Compared to the wild-type and *cat2-1*, the *sid2-2* and *cat2-1 sid2-2* plants subjected to *Pst* DC3000 infection had much lower levels of SA and SAG (SA-2-*O*-β-D-Glucoside) (Figure 1D). Upon pathogen infection, *sid2-2* accumulated more IAA and showed greater susceptibility to the pathogen than the wild-type (Figures 1C and 1E), suggesting that SA negatively regulates IAA levels for pathogen resistance. Furthermore, the *cat2-1 sid2-2* double mutant had less IAA and higher resistance to *Pst* DC3000 than *sid2-2* single mutant. Taken together, our data suggest that SA plays its role partially through catalase-mediated changes in IAA accumulation.

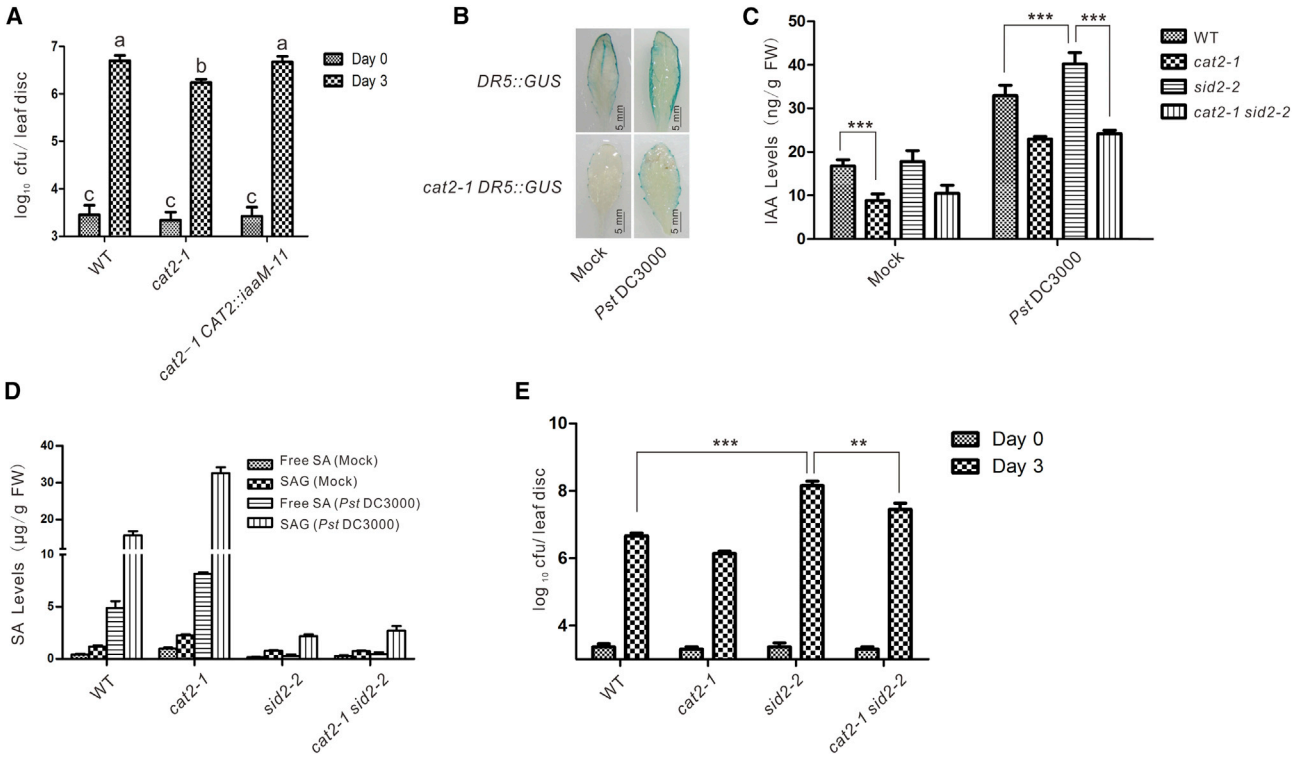

Figure 1. SA Modulates Plant Defense Response to *Pst* DC3000 through Catalase-Mediated Change of IAA Accumulation

(A) Bacterial growth was measured at day 0 and day 3 after the wild-type, *cat2-1*, and *cat2-1 CAT2::iaaM-11* plants were infected with *Pst* DC3000.

(B) GUS staining for GUS activity was performed with *Pst* DC3000-infected and mock-treated *DR5::GUS and cat2-1 DR5::GUS* plants at 72 hr after inoculation.

(C and D) The wild-type, *cat2-1*, *sid2-2*, and *cat2-1 sid2-2* plants were infected with *Pst* DC3000. The infected and mock-treated leaves were collected at 3 days after inoculation and used to measure IAA (C), SA (D), and SAG (D). FW indicates fresh weight.

(E) Bacterial growth in the wild-type, *cat2-1*, *sid2-2*, and *cat2-1 sid2-2* plants was assessed at 3 days after inoculation.

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks represents statistical significance (Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars with different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's multiple comparison test. See also Figure S7.

SA Antagonizes IAA Accumulation by Inhibiting CAT2 Activity to Increase H_2O_2 Levels

We further explored CAT2 functions in SA-mediated changes in IAA accumulation in response to pathogen infection. SA can bind CAT proteins and inhibit their activity, resulting in increased levels of H_2O_2 (Chen et al., 1993; Conrath et al., 1995). Thus, the pathogen-mediated increase in SA could repress IAA accumulation by inhibiting CAT2 activity via direct interaction. To test it, total proteins isolated from the leaves of both wild-type and *sid2-2* plants treated with or without *Pst* DC3000 and SA were used for CAT activity assay. While all of these materials had similar accumulation of CAT proteins detected with anti-CAT2 and anti-CAT1 antibodies (Figure S1), CAT activity was dramatically reduced in wild-type *Arabidopsis* treated with either *Pst* DC3000 or SA (Figure 2A). Consistent with the decreased CAT activity, H_2O_2 levels increased in the treated plants (Figure 2B), implying that SA functions in the regulation of CAT activity to induce changes in H_2O_2 levels. In addition, the *Pst* DC3000-mediated suppression of CAT activity observed in the wild-type was compromised in the *sid2-2* mutant (Figure 2A). Similarly, H_2O_2 was much higher in wild-type than in *sid2-2* when challenged with *Pst* DC3000 (Figure 2B). Exogenous SA rescued the pathogen-induced changes of CAT activity and H_2O_2 levels in *sid2-2* plants (Figures 2A and 2B). These results further suggest that SA is required for *Pst* DC3000-induced suppression of CAT activity and increase of H_2O_2 level in plants.

Then, we assayed whether SA inhibited CAT2 activity using purified CAT2 protein expressed in *E. coli*. Indeed, SA decreased CAT2 activity in a dose-dependent manner (Figure 2C). To verify the role of CAT2 in SA-mediated inhibition of CAT activity in plants, we measured CAT activities in SA-treated plants. SA treatment decreased CAT activities in both the wild-type and *cat2-1*, but *cat2-1* was less sensitive to SA in terms of changes in CAT activities than the wild-type (Figure 2D), suggesting that CAT2 is a target of SA-mediated suppression of CAT activity in plants. Taken together with the above data that SA functions in catalase-mediated changes in IAA accumulation, these results show that SA antagonizes IAA accumulation by increasing H_2O_2 levels via inhibition of CAT2 activity.

H_2O_2 Reduces IAA Accumulation by Sulfenylating TSB1 on Cysteine 308 to Decrease TSB1 Activity

We next explored whether H_2O_2 modulates IAA levels through sulfenylation of target proteins in a similar way to the action of H_2O_2 in mammals and microbes (Karisch et al., 2011; Kim et al., 2002). We assayed sulfenylated proteins in *Arabidopsis* via the biotin-switch method used in mammals and microbes

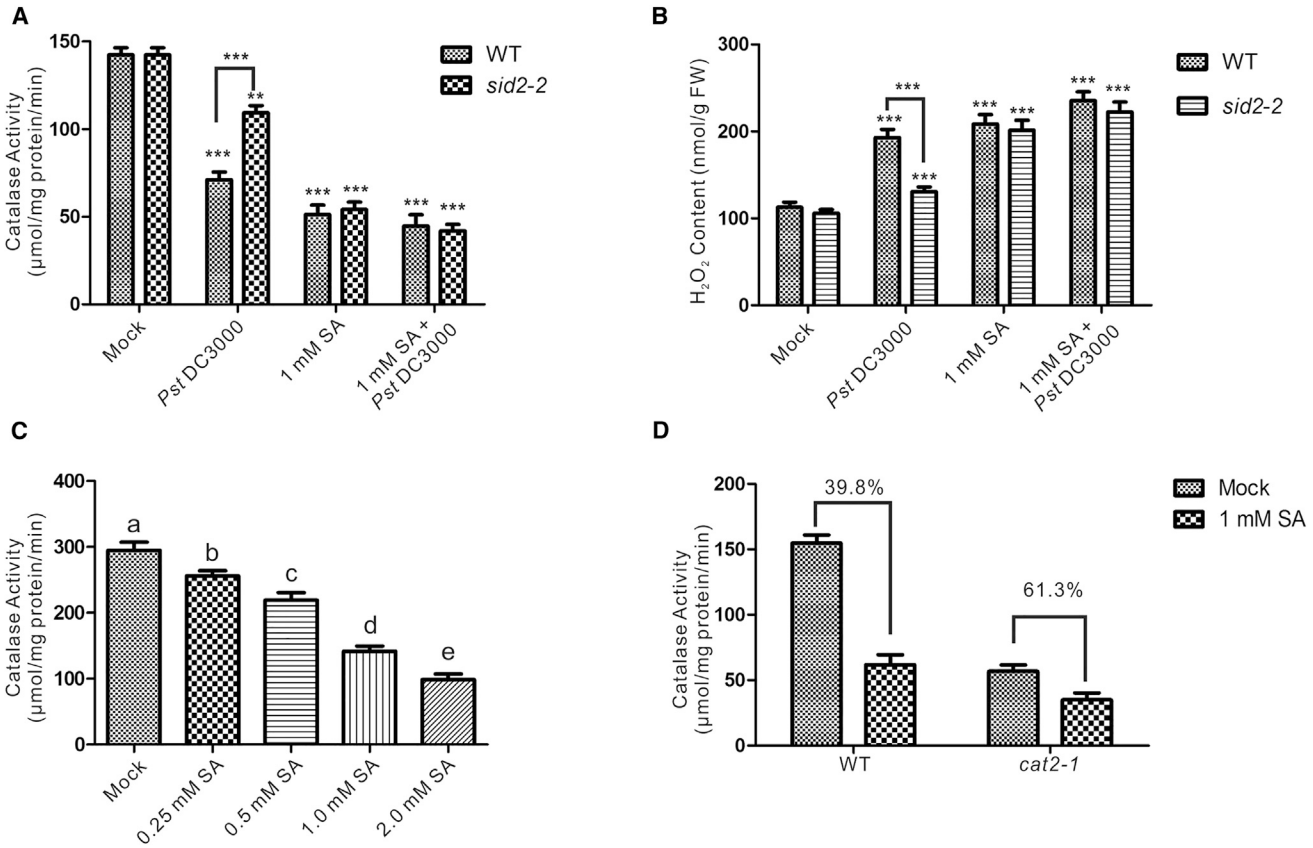

Figure 2. SA Increases H_2O_2 Content by Inhibiting CAT2 Activity

(A and B) The leaves of the wild-type and *sid2-2* plants 3 days after the indicated treatments were collected and used to measure the catalase activity (A) and H_2O_2 content (B).

(C) The activities of purified CAT2 protein were assayed in the reactions with various SA concentrations. Different letters indicate significant differences between treatments ($p < 0.05$ by one-way ANOVA with Tukey's multiple comparison test).

(D) Catalase activities of the wild-type and *cat2-1* plants treated with or without SA were measured.

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks indicate significant differences compared to mocks with same genotype (A and B) (Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$). See also Figure S1.

(Kim et al., 2002; Saurin et al., 2004). One sulfenylated protein, tryptophan (Trp) synthetase β subunit 1 (TSB1), was further investigated, since TSB1 functions in IAA biosynthesis and IAA is involved in SA-mediated disease-resistance (Wang et al., 2007). Treatment with 10 and 100 μM H_2O_2 led to a higher degree of sulfenylation in purified *Arabidopsis* TSB1 expressed in *E. coli* than in the untreated control (Figure 3A). The observed decrease in TSB1 sulfenylation at 1 and 10 mM H_2O_2 is likely due to sulfinic acid or sulfonic acid modification of the protein (Figure 3A), as previously reported (Saurin et al., 2004). To further confirm TSB1 sulfenylation in vivo, total sulfenylated proteins isolated from the leaves of wild-type and *cat2-1* plants were separated by SDS-PAGE, and sulfenylated TSB1 was detected by immunoblot with an antibody against TSB1. Although wild-type and *cat2-1* plants had similar accumulation of TSB1 protein (Figure S1), TSB1 showed significantly more sulfenic acid modification in *cat2-1* than the wild-type (Figure 3B), indicating that enhanced H_2O_2 accumulation promotes sulfenic acid modification of TSB1.

We hypothesized that sulfenic acid modification might affect TSB1 enzymatic activity. Indeed, H_2O_2 significantly suppressed TSB1 activity in vitro in a dose-dependent manner (Figure 3C). Furthermore, the leaves of *cat2-1* plants exhibited only about half of the TSB activity found in the wild-type (Figure 3D). This decreased TSB activity also resulted in low Trp contents in *cat2-1* compared with the wild-type (Figure 3E). Taking these data together with the observation of decreased IAA accumulation in *cat2-1* (Figure 3F), we conclude that the decreased IAA in *cat2-1* is due to the decrease in the IAA precursor Trp. Indeed, exogenous Trp rescued decreased IAA in *cat2-1* (Figure 3F). Additionally, overexpressing *TSB1* in *cat2-1* increased the Trp contents and IAA accumulation in *cat2-1 35S::TSB1* to levels similar to those in the wild-type (Figures 3E, 3F, and S2).

To further dissect the roles of the five cysteine (Cys) residues of TSB1 in sulfenic acid modification for changes in TSB1 activity, we expressed and purified mutated TSB1 proteins (TSB1-C53S, TSB1-C201S, TSB1-C208S, TSB1-C308S, and TSB1-C440S) in which each Cys of the five was individually replaced with serine via site-directed mutagenesis. Each mutated TSB1 except TSB1-C201S exhibited reduced enzymatic activity compared with TSB1 (Figure 3G). Also, H_2O_2

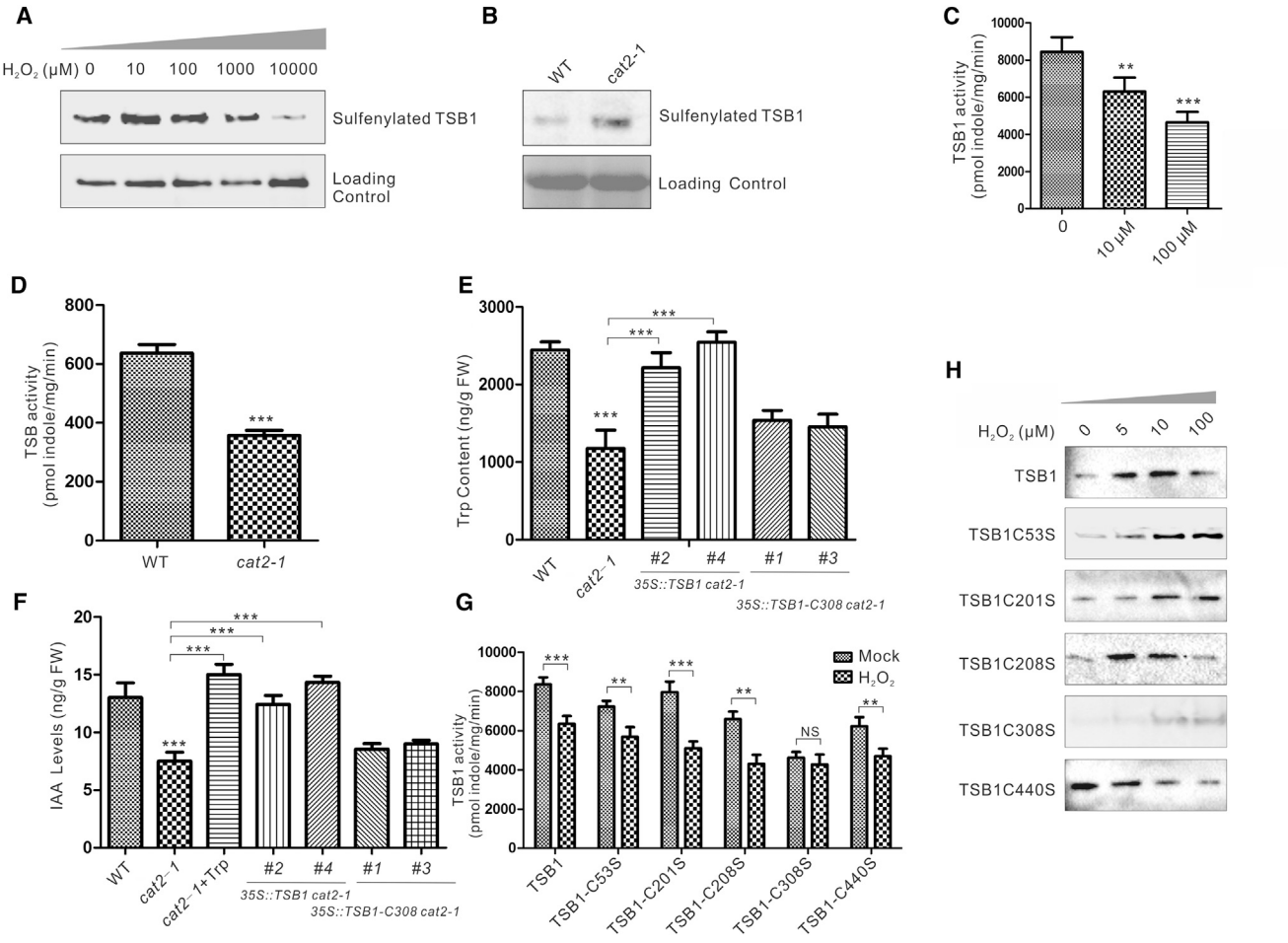

Figure 3. H_2O_2 Decreases IAA Accumulation by Sulfenylating TSB1

(A) Sulfenylated TSB1 was assayed by western blotting after purified TSB1 protein was treated with different concentrations of H_2O_2 for 30 min.

(B) TSB1 sulfenylation was assayed in both wild-type and *cat2-1* plants.

(C) Activities of purified TSB1 were measured in the presence of H_2O_2 at different concentrations.

(D) TSB activity was measured in both wild-type and *cat2-1* plants.

(E) Tryptophan (Trp) content in wild-type, *cat2-1*, *35S::TSB1 cat2-1*, and *35S::TSB1C308S cat2-1* plants.

(F) IAA levels in wild-type, *cat2-1*, Trp-treated *cat2-1*, *35S::TSB1 cat2-1*, and *35S::TSB1C308S cat2-1* plants.

(G) Activities of purified TSB1, TSB1C53S, TSB1C201S, TSB1C208S, TSB1C308S, and TSB1C440S were measured in the absence or presence of H_2O_2.

(H) Sulfenylated TSB1, TSB1C53S, TSB1C201S, TSB1C208S, TSB1C308S, and TSB1C440S were assayed by western blotting after these proteins were treated with H_2O_2 at different concentrations for 30 min.

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks indicate significant differences compared to untreated control (C) or wild-type (E and F) (Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$). See also Figures S2 and S6.

treatment significantly decreased the activity of TSB1-C53S, TSB1-C201S, TSB1-C208S, and TSB1-C440S, but not TSB1-C308S (Figure 3G), suggesting the importance of Cys308 in H_2O_2-mediated inhibition of TSB1 activity. Consistent with this, TSB1-C308S was less sensitive to H_2O_2 in terms of changes of sulfenic acid modification than TSB1, TSB1-C53S, TSB1-C201S, and TSB1-C208S (Figure 3H). The importance of Cys308 was also supported by transgenic analysis showing that overexpressing TSB1, but not TSB1-C308S, rescued the reduced Trp content and the IAA accumulation in *cat2-1* (Figures 4E and 4F). The observed higher sensitivity of TSB1-C440S to H_2O_2-mediated sulfenylation may be due to mutation-induced conformational changes (Figure 3H).

TSB1 Is Involved in SA-Mediated Defense against *Pst* DC3000

The above data indicated that pathogen-induced SA repressed IAA accumulation by inhibiting CAT2 activity to increase H_2O_2 contents and that TSB1 participates in the regulation of IAA accumulation through H_2O_2-mediated sulfenylation. We further assessed the possible role of TSB1 in SA-mediated pathogen resistance. First, we assayed both TSB activity and Trp contents in *Arabidopsis* plants with or without *Pst* DC3000 infection. We found that *Pst* DC3000 infection increased Trp contents by promoting TSB activity in wild-type compared with uninfected plants, consistent with our above data (Figure 1C) and previous reports that *Pst* DC3000 infection can increase IAA level for

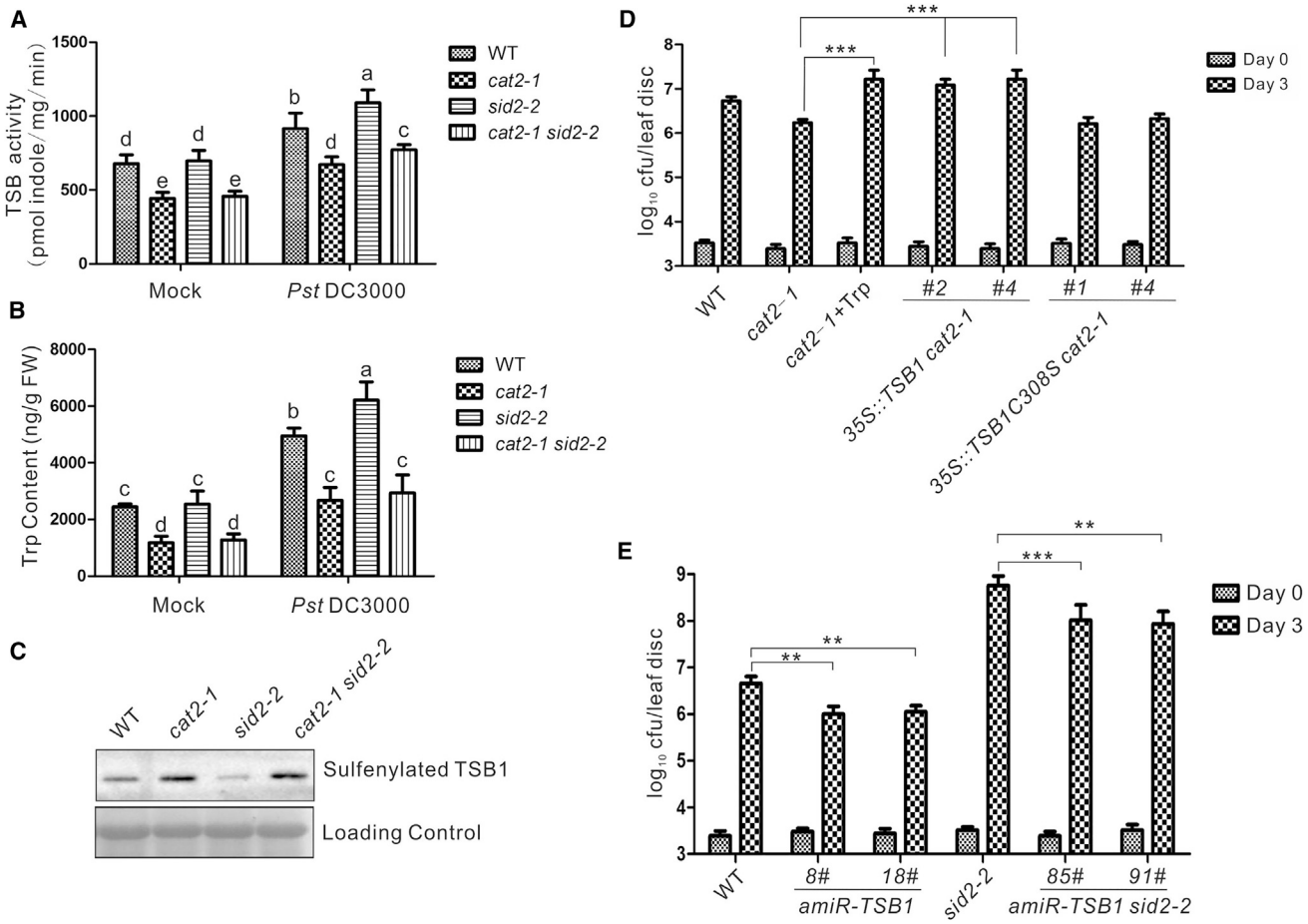

Figure 4. TSB1 Involves SA-Mediated Defense against *Pst* DC3000 by Regulating Tryptophan Accumulation

(A and B) TSB activities (A) and Trp content (B) were measured in wild-type, *cat2-1*, *sid2-2*, and *cat2-1 sid2-2* plants challenged with or without *Pst* DC3000.

(C) TSB1 sulfenylation was measured in wild-type, *cat2-1*, *sid2-2*, and *cat2-1 sid2-2* plants after *Pst* DC3000 infection.

(D) Bacterial growth in wild-type, *cat2-1*, Trp-treated *cat2-1*, *35S::TSB1 cat2-1*, and *35S::TSB1C308S cat2-1* plants was assessed at 3 days after inoculation.

(E) Bacterial growth in wild-type, *amiR-TSB1*, *sid2-2*, and *amiR-TSB1 sid2-2* plants was assessed at 3 days after inoculation.

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks indicate significant differences by Student's t test (**$p < 0.01$, ***$p < 0.001$). Bars with different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's multiple comparison test. See also Figures S1 and S2.

invasion (Kazan and Manners, 2009; Figures 4A and 4B). These increases were also assayed in *cat2-1* plants in comparison with uninfected control (Figures 4A and 4B). However, both TSB activity and Trp contents in *cat2-1* were significantly lower than those in wild-type upon *Pst* DC3000 infection (Figures 4A and 4B). Consistently, TSB1 sulfenylation was much higher in pathogen-infected *cat2-1* than in infected wild-type (Figure 4C), although the infected wild-type and *cat2-1* plants had similar accumulation of TSB1 protein (Figure S1). Taken together, our data suggest that H_2O_2-mediated TSB1 sulfenylation represses *Pst* DC3000-induced TSB activity and Trp accumulation. Considering the decreased IAA accumulation and increased resistance of *cat2-1* plants, which have higher H_2O_2 contents (Figures 1 and 4D), these data also imply that the reduced TSB activity and Trp accumulation in *cat2-1* suppress IAA accumulation and thus enhance the pathogen resistance of the mutant plants. This notion was further supported by our observation of enhanced IAA accumulation, and thus reduced resistance, of Trp-treated *cat2-1* (Figures 3F and 4D). In addition, the higher resistance of *cat2-1* could be compromised by overexpressing *TSB1* for higher Trp and IAA accumulation, but not by overexpressing *TSB1-C308S*, in which TSB1 activity was markedly repressed (Figures 3E–3G and 4D).

We then examined whether SA was involved in the regulation of TSB activity and Trp contents in response to *Pst* DC3000 infection by using *sid2-2* and *cat2-1 sid2-2* mutants. *Pst* DC3000 infection significantly stimulated TSB activity and Trp accumulation in *sid2-2* compared with the wild-type (Figures 4A and 4B), indicating that SA acts negatively in this stimulation. SA may participate in this process by elevating H_2O_2 accumulation via inhibition of CAT2. As expected, the *Pst* DC3000-stimulated TSB activity and Trp accumulation were suppressed in *cat2-1 sid2-2* compared with *sid2-2*, which has lower H_2O_2 content (Figures 2B, 4A, and 4B). Additionally, more TSB1 was sulfenylated in *Pst* DC3000-infected *cat2-1 sid2-2* than *sid2-2*, although these infected mutants accumulated similar levels of

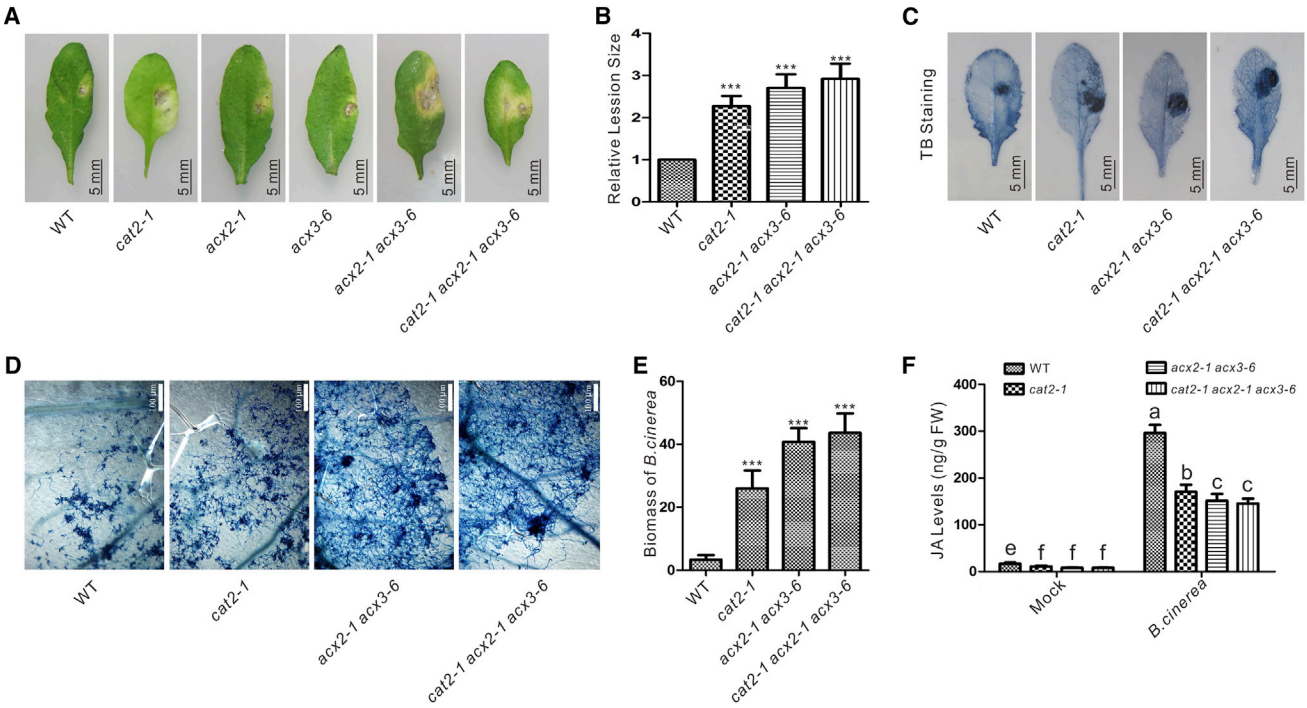

Figure 5. The *cat2-1*, *acx2-1 acx3-6*, and *cat2-1 acx2-1 acx3-6* Mutants Exhibit Increased Susceptibility to *B. cinerea*

(A) The photographs were taken with the leaves of the wild-type, *cat2-1*, *acx2-1*, *acx3-6*, *acx2-1 acx3-6*, and *cat2-1 acx2-1 acx3-6* plants at 3 days after infection with *B.cinerea*.

(B–E) Relative lesion size (B), photos (C) and microscopic photos (D) of Typan-blue staining, and biomass of *B.cinerea* (E) were shown in the leaves of the wild-type, *cat2-1*, *acx2-1 acx3-6*, and *cat2-1 acx2-1 acx3-6* plants at 3 days after infection with *B.cinerea*.

(F) JA levels were measured in the leaves of the wild-type, *cat2-1*, *acx2-1 acx3-6*, and *cat2-1 acx2-1 acx3-6* plants at 3 days after inoculation with *B.cinerea*.

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks represents significant differences compared to wild-type (Student's t test, ***$p < 0.001$). Bars with different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's multiple comparison test. See also Figure S7.

TSB1 protein (Figures 4C and S1), implying that H_2O_2-mediated TSB1 sulfenylation affects Trp accumulation by repressing TSB1 activity. Taken together with our above data that SA modulates plant immune responses by inhibiting CAT2 to increase H_2O_2 contents and cause lower IAA accumulation, we conclude that TSB1 functions in SA-mediated defense against *Pst* DC3000 by regulating the accumulation of tryptophan as an IAA precursor through H_2O_2-mediated TSB1 sulfenylation resulting from SA-mediated inhibition of CAT2. We also obtained transgenic *amiR-TSB1* and *amiR-TSB1 sid2-2* plants with an artificial microRNA (amiR) approach and assayed their resistance to *Pst* DC3000. Consistent with the above data, reduced *TSB1* expression in *amiR-TSB1* and *amiR-TSB1 sid2-2* conferred enhanced resistance to *Pst* DC3000 compared with either the wild-type or *sid2-2*, respectively (Figures 4E and S2).

The *cat2-1* Mutant Exhibits Increased Susceptibility to *B. cinerea* and Decreased JA Levels

JA and SA act antagonistically to mediate defense responses against specific types of pathogens. For example, *coi1* and *myc2/jin1* are more resistant to the biotrophic pathogen *Pst* DC3000, but more susceptible to the necrotrophic pathogen *B. cinerea*, than the wild-type (Laurie-Berry et al., 2006). To assess the resistance of *cat2-1* to necrotrophic pathogens, we challenged the wild-type and *cat2-1* with *B. cinerea* and observed increased susceptibility to *B. cinerea* in *cat2-1* (Figure 5). Compared with the wild-type, *cat2-1* displayed larger lesions and more extensive pathogen growth with more fungal biomass (Figures 5A–5E).

Plant resistance to the necrotrophic pathogen *B. cinerea* requires JA and JA signaling. Therefore, we directly measured JA in *cat2-1*. Although *B. cinerea*-infected *cat2-1* and wild-type plants accumulated more JA than uninfected plants, JA levels in infected *cat2-1* were much lower than those in the wild-type challenged with *B. cinerea* (Figure 5F), consistent with the visual observation that *cat2-1* was more sensitive to *B. cinerea*. These results indicated that *cat2-1* exhibited enhanced susceptibility to *B. cinerea* along with decreased JA levels. Taken together with the above data that CAT2 acts in SA-mediated resistance to *Pst* DC3000, these results suggest that CAT2 may function in SA-mediated antagonistic effects on JA.

CAT2 Promotes the Activity of ACX2/ACX3 through Direct Interaction

We carried out yeast two-hybrid screening to search for CAT2-interacting proteins and unexpectedly identified the peroxisomal JA-biosynthetic enzymes ACX2 and ACX3 (Figure 6A; Li et al., 2005). This allowed us to address how SA modulates JA metabolism, a phenomenon reported more than two decades ago, although the underlying mechanism has remained unclear.

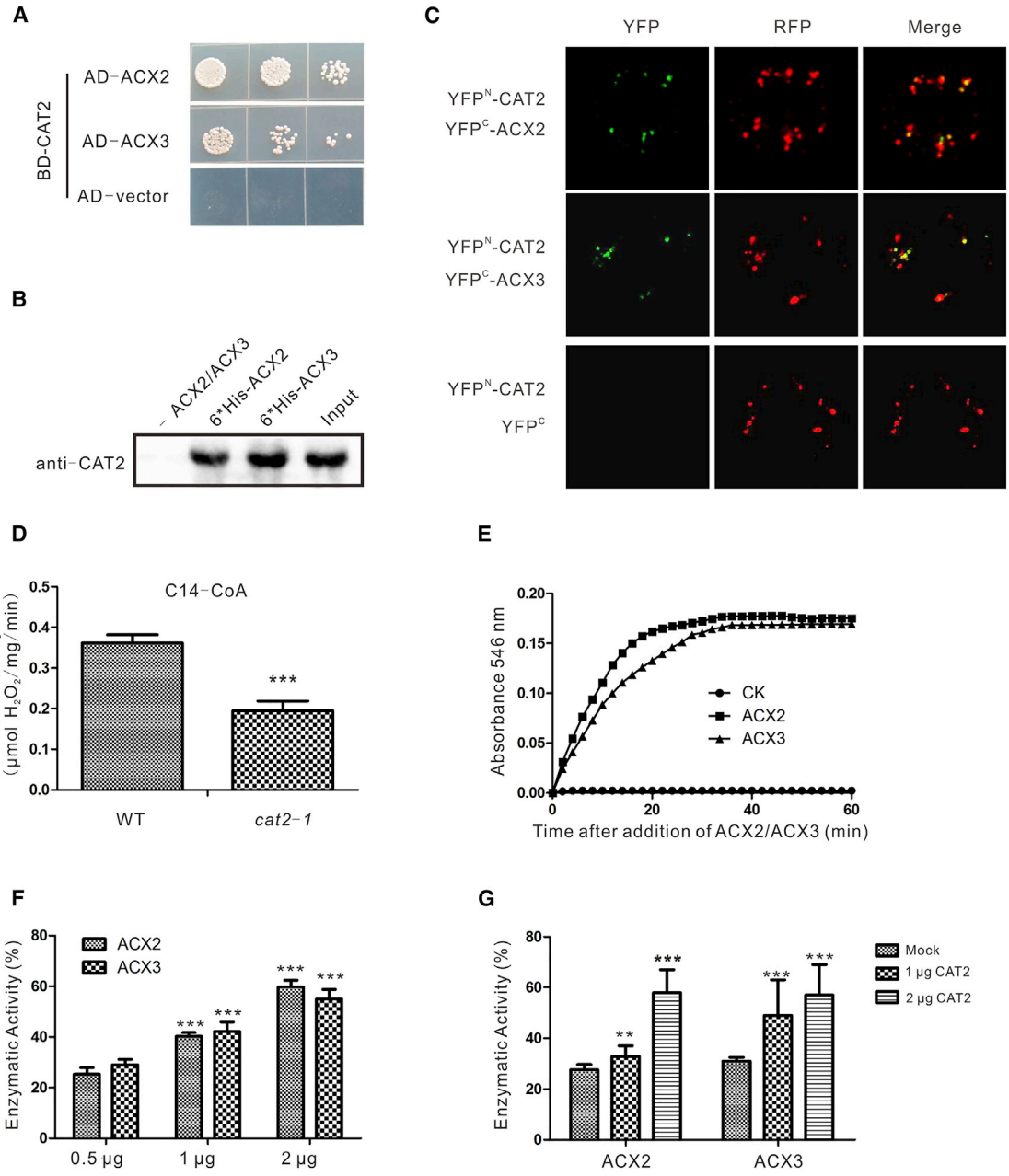

Figure 6. CAT2 Promotes the Enzymatic Activities of ACX2/ACX3 Through Their Interaction

(A–C) CAT2 interacted with both ACX2 and ACX3 assayed with the yeast two-hybrid system (A), pull-down experiments (B), and BiFC technique (C). Red dots in (C) indicate the peroxisomes.

(D) ACX activities of the wild-type and *cat2-1* plants were assessed with C14-CoA as substrate.

(E) The activities of purified ACX2 and ACX3 were assayed with OPC4-CoA as substrate. H_2O_2 production was monitored as ACX2/ACX3 activities as described in Experimental Procedures.

(F and G) ACX2/ACX3 activities in the absence (F) or presence (G) of CAT2 were measured with OPC4-CoA as substrate by using MALDI TOF-MS. ACX2/ACX3 activities were defined as the ratio of Δ^2-OPC4-CoA to (Δ^2-OPC4-CoA + OPC4-CoA).

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks represent significant differences compared to wild-type (D) or same protein at 0.5 μg (F and G) (Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$). See also Figures S3 and S4.

To verify the interaction between CAT2 and ACX2/ACX3, a pull-down assay was performed. Total proteins isolated from wild-type leaves were incubated with or without 6× His-tagged ACX2 or ACX3 and precipitated with agarose-conjugated anti-His-tag antibody. The precipitants were separated by SDS-PAGE and immunoblotted with anti-CAT2 antibody. Our results indicated that both ACX2 and ACX3 interacted with CAT2 (Figure 6B). Additional evidence for the interaction of these proteins

came from yellow fluorescent protein (YFP)-based biomolecular fluorescence complementation (BiFC) assays. Constructs for the expression of YFP^N-CAT2, YFP^C-ACX2, and YFP^C-ACX3 were introduced into protoplasts of lines expressing the peroxisomal marker *PX-RK* (Nelson et al., 2007). YFP fluorescence was absent in the negative control, but protoplasts coexpressing YFP^N-CAT2 and either YFP^C-ACX2 or YFP^C-ACX3 showed reconstituted YFP signal (Figure 6C), indicating that CAT2 directly interacted with ACX2 and ACX3 in peroxisomes.

Mutation of tomato *ACX1A* results in decreased JA (Li et al., 2005). Accordingly, we assayed the susceptibility of *Arabidopsis acx2-1* and *acx3-6* mutants to *B. cinerea*. The *acx2-1* and *acx3-6* mutants displayed resistance to *B. cinerea* similar to the wild-type (Figure 5A). However, the *acx2-1 acx3-6* double mutant showed increased susceptibility, as indicated by larger lesions and more extensive growth, with higher biomass of *B. cinerea* than the wild-type (Figures 5A–5E). In agreement with these results, *acx2-1 acx3-6* had much lower JA level than the wild-type when subjected to pathogen infection (Figure 5F). In addition, *cat2-1 acx2-1 acx3-6* triple mutant exhibited susceptibility to *B. cinerea* and JA accumulation similar to that of *acx2-1 acx3-6* (Figure 5), suggesting that CAT2 affects plant resistance to *B. cinerea* through ACX2/ACX3. Together, these data confirmed that, similar to *cat2-1*, *acx2-1 acx3-6* exhibited susceptibility to *B. cinerea* along with decreased JA level.

Next, we examined whether the reduced JA accumulation in *cat2-1* was due to the lack of CAT2 stimulation of ACX2/ACX3 activity in JA biosynthesis. Previous biochemical evidence indicated that the ACX isoenzymes have overlapping but distinct substrate specificities (Li et al., 2005); therefore, we assayed ACX activity with C8-CoA, C12-CoA, C14-CoA, C16-CoA, and C18-CoA as substrates. The ACX activities of wild-type and *cat2-1* plants exhibited small differences with C8-CoA, C12-CoA, C16-CoA and C18-CoA (Figure S3), but we observed a dramatic reduction of ACX activity in *cat2-1* compared with the wild-type when we used C14-CoA as the substrate (Figure 6D); this suggests that ACX activity requires CAT2, especially in catalyzing the dehydrogenation of C14-CoA. Thus, OPC4-CoA was employed as substrate in our further experiments to assay ACX2/ACX3 activity for OPC4-CoA β-oxidation in JA biosynthesis because OPC4-CoA and C14-CoA have an equal number of carbon atoms. Purified ACX2/ACX3 efficiently catalyzed dehydrogenation of OPC4-CoA to produce Δ^2-OPC4-CoA and H_2O_2 in the assays based on H_2O_2-coupled color reaction (Figure 6E; Li et al., 2005). However, the H_2O_2-coupled color reaction cannot be used to test the effect of CAT2 on the reaction because CAT2 scavenges H_2O_2. Therefore, we used MALDI TOF-MS in positive-ion mode to directly measure the relative contents of the substrate OPC4-CoA and product Δ^2-OPC4-CoA (Figure S4) and defined the enzymatic activity of ACX as the ratio of Δ^2-OPC4-CoA to (Δ^2-OPC4-CoA + OPC4-CoA). The enzymatic activities increased as more ACX2 or ACX3 was added into the reaction without CAT2 (Figure 6F). The addition of CAT2 markedly stimulated ACX2/ACX3 activity in a dose-dependent manner (Figure 6G). Taken together with our above data that both *cat2-1* and *acx2-1 acx3-6* were susceptible to *B. cinerea* and had decreased JA accumulation, these results suggest that CAT2 promotes the activities of ACX2/ACX3 through interaction with these two enzymes for JA biosynthesis.

SA Represses JA Accumulation Via Its Interaction with CAT2 to Reduce ACX2/ACX3 Activity in Plant Defense Responses

Our observations that SA inhibited CAT2 activity and that CAT2 stimulated ACX2/ACX3 activity prompted us to explore whether SA antagonizes JA biosynthesis by inhibiting CAT to impair ACX2/ACX3 activity for differential plant defense responses to infection with biotrophic and necrotrophic pathogens. First, we assessed the effect of SA on CAT2-mediated stimulation of ACX2/ACX3 activities. We found that SA alone did not affect the enzymatic activity of ACX2 or ACX3 in the reaction without CAT2, but SA significantly repressed CAT2-promoted enzymatic activities of ACX2 or ACX3 (Figure 7A). Thus, physical interaction between CAT2 and ACX2/ACX3 may be affected by SA. To assess this, we performed coimmunoprecipitation (coIP) assay with *35S::GFP-ACX2* and *35S::GFP-ACX3* plants treated with or without SA. The anti-GFP antibody-precipitated proteins were separated by SDS-PAGE and immunoblotted with anti-CAT2 antibody. Our results indicated that ACX2/ACX3 pulled down CAT2 in vivo, but the CAT2 amount pulled down by anti-GFP antibody was reduced in the presence of SA, revealing that SA represses the physical interaction between CAT2 and ACX2/ACX3 (Figure 7B). These results together suggested that SA can inhibit ACX2/ACX3 activities by affecting the interaction between CAT2 and ACX2/ACX3. Then, we examined the susceptibility of the wild-type and *cat2-1* plants to *B. cinerea* by applying SA. Consistent with the previous report that SA-treated wild-type plants are more sensitive to the necrotrophic pathogen *A. brassicicola* and have reduced JA signaling (Spoel et al., 2007), wild-type and *cat2-1* plants exhibited increased sensitivity to *B. cinerea* in the presence of SA compared with untreated control (Figure 7C). However, SA treatment increased the susceptibility of *cat2-1* to *B. cinerea* by only 28.7%, whereas it increased the susceptibility of the wild-type to the pathogen by 320% (Figure 7C). These results could be due to SA repressing JA accumulation via its interaction with CAT2 to decrease ACX2/ACX3 activities in SA-treated wild-type, whereas *CAT2* was mutated in *cat2-1*, leading to less of an effect in the mutant background. Indeed, SA treatment markedly suppressed *B. cinerea*-induced ACX activities and JA accumulation in the wild-type, but not in *cat2-1* (Figures 7D and 7E).

We further monitored ACX activities and JA levels in *sid2-2* and *cat2-1 sid2-2* because *Pst* DC3000-induced SA biosynthesis is repressed in the mutants due to the mutation of the SA biosynthesis gene *ICS1*. JA levels dramatically increased in response to *Pst* DC3000 infection in a mutant that fails to accumulate SA (Spoel et al., 2003). Similarly, both *sid2-2* and *cat2-1 sid2-2* accumulated much more JA than both the wild-type and *cat2-1* when infected with *Pst* DC3000 (Figure 7F). Consistent with these findings, these two mutants also exhibited enhanced ACX activity upon *Pst* DC3000 infection (Figure 7G). These results supported the conclusion that SA antagonizes both ACX activity and JA accumulation because infection dramatically induced SA in the wild-type and *cat2-1*, but not in *sid2-2* and *cat2-1 sid2-2*. This stimulation of ACX activity and JA levels was much weaker in *cat2-1 sid2-2* than in *sid2-2* (Figures 7F and 7G), suggesting that ACX activity and JA accumulation upon pathogen infection require CAT2. Taken together, our results showed that SA accumulation induced by the biotrophic

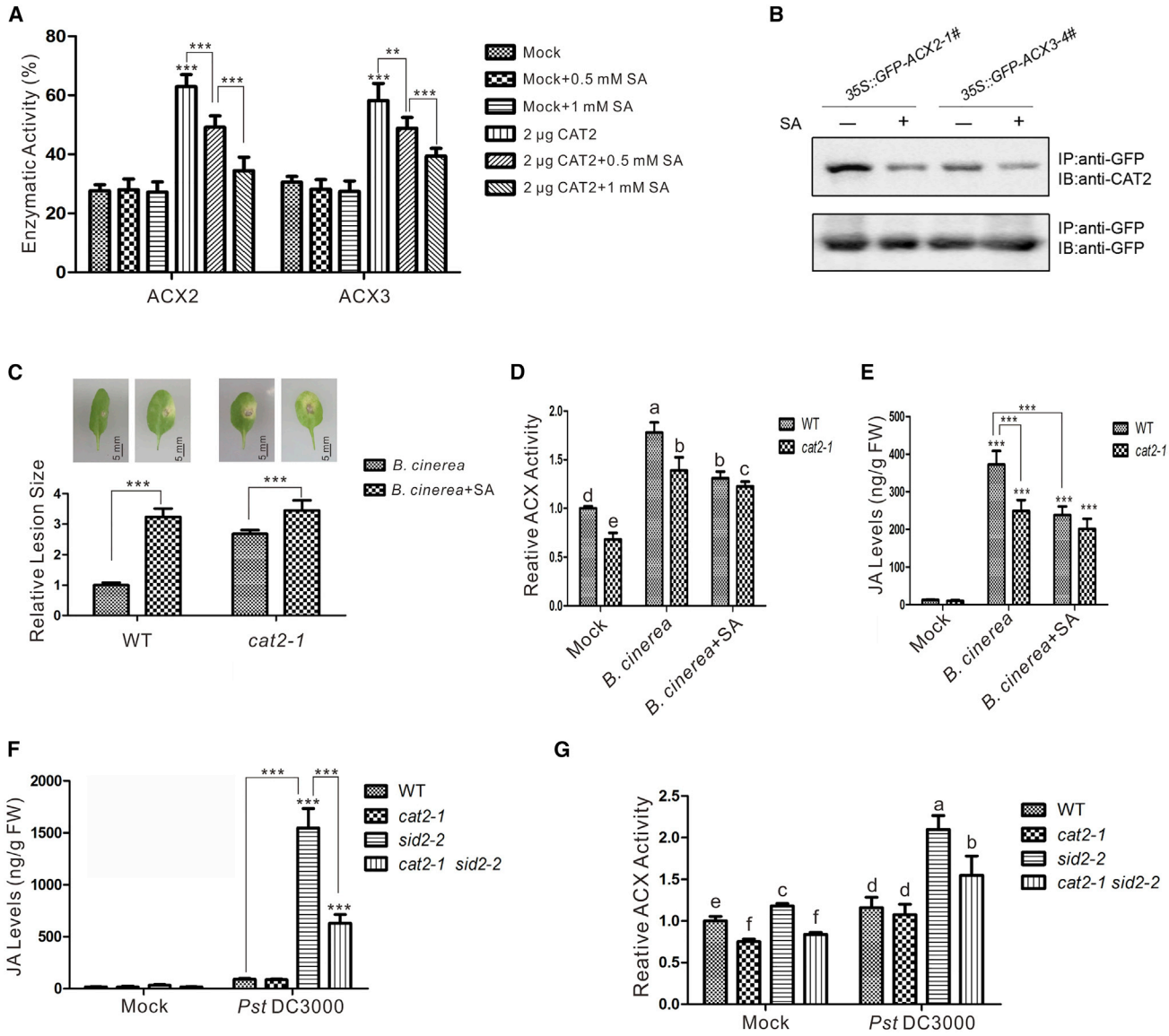

Figure 7. SA Represses JA Accumulation by Reducing CAT2-Promoted ACX2/ACX3 Activity

(A) ACX2/ACX3 enzymatic activities were assessed with OPC4-CoA as substrate under indicated conditions.

(B) CoIP assays examine the effect of SA on the interaction between CAT2 with ACX2/ACX3 in plants. Total proteins were extracted from 10-day-old *35S::GFP-ACX2* or *35S::GFP-ACX3* seedlings treated with or without 1 mM SA for 12 hr and immunoprecipitated with anti-GFP antibody-conjugated agarose. The precipitants were separated by SDS-PAGE and assayed with anti-CAT2 antibody.

(C) Both the wild-type and *cat2-1* plants were pretreated with water or SA for 1 day and then inoculated with *B. cinerea*. At 3 days after inoculation, photographs of the disease symptoms were taken (top) and relative lesion size was shown (bottom).

(D and E) Both the wild-type and *cat2-1* plants were pretreated with water or SA for 1 day and then inoculated with *B. cinerea*. The leaves were collected to measure relative ACX activities (D) and JA levels (E) at 3 days after inoculation.

(F and G) JA levels (F) and relative ACX activities (G) in the wild-type, *cat2-1*, *sid2-2*, and *cat2-1 sid2-2* plants were measured at 3 days after *Pst* DC3000 inoculation.

All experiments were repeated at least three times. Data are shown as means ± SEM. Asterisks represents significant differences in comparison with same protein (A) or mock in the same genotype (E and F) (Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars with different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's multiple comparison test. See also Figure S5.

pathogen *Pst* DC3000 could suppress CAT2-mediated stimulation of ACX2/ACX3 activity and decrease JA levels.

In summary, our study shows how CAT2 coordinates SA repression of auxin accumulation and JA biosynthesis in plant defense against pathogen infection. CAT2 manipulates SA-mediated repression of auxin accumulation by changing Trp accumulation through H_2O_2-mediated sulfenylation of TSB1, and it suppresses JA biosynthesis via changes in ACX2/ACX3 activity through direct interaction with these enzymes (Figure S5).

DISCUSSION

Long-term coevolution of plants and pathogens has produced various defense strategies involving complex signaling networks of plant hormones (Spoel and Dong, 2008). Crosstalk between different hormone-mediated defense pathways can optimize plant responses to activate the most suitable defense strategies against specific invaders.

Auxin modulates plant growth by promoting cell elongation and expansion. However, rapid growth often causes negative effects such as reduced resistance to biotrophic pathogens (Kazan and Manners, 2009). Indeed, many pathogens hijack the plant auxin signaling system to promote virulence. For example, biotrophic pathogens can synthesize and release auxin or auxin analogs into the host cells. Pathogens can also enhance plant auxin responses by effector-stimulated turnover of auxin/IAA (AUX/IAA) proteins (Cui et al., 2013). However, coevolution of plants and pathogens led to suppressed auxin signaling in plants via SA-promoted stability of AUX/IAA proteins (Wang et al., 2007). The repressed auxin signaling could be due to decreased IAA levels. Here we illustrate that pathogen-induced SA reduces auxin levels to enhance disease resistance by increasing the accumulation of H_2O_2 as SA represses CAT activity through its interaction with CAT2.

ROS participate in plant development and responses to various environmental stresses, including abiotic stresses and defense against pathogens (Torres et al., 2006). However, how ROS function in plants to mediate such different processes remains unclear. Here, we identified sulfenylated plant proteins and showed that one of these proteins, TSB1, is required in SA-mediated plant disease resistance to reduce IAA accumulation. Our data also revealed that H_2O_2 functions in this process by sulfenylating the conserved Cys 308 of TSB1 (Figure S6), suggesting that this mechanism of H_2O_2-mediated repression of Trp accumulation may exist in different species. In addition, our data indicating that the sulfenylation of Cys 308 affected TSB1 activity are consistent with a previous report, which demonstrated that a hydrogen bond formed by Cys 308 and an adjacent serine is required for the binding of coenzyme pyridoxal 5-phosphate to the TSB active site (Hioki et al., 2004).

SA and JA signaling pathways are activated to cope with biotrophic and necrotrophic pathogens, respectively. Earlier studies documented the antagonistic interactions between SA and JA, although the underlying mechanisms remained to be explored (Van der Does et al., 2013). Recently, Zheng et al. (2012) showed that biotroph-secreted COR represses SA accumulation by modulating the expression of genes involved in SA biosynthesis and conjugation. However, how SA modulates JA metabolism remained unknown. Here, our study revealed that SA represses JA accumulation through its interaction with CAT2, which is required to stimulate ACX2/ACX3 activity for JA biosynthesis. Thus, CAT2 plays a vital role in SA's antagonism of JA. The greater *Pst* DC3000-induced increase of ACX activity and JA levels in *sid2-2* compared with the wild-type was not totally abolished in *cat2-1 sid2-2* (Figures 7F and 7G). This finding could indicate that other factors may also participate in this process.

We also tested whether mutations in *CAT1* and *CAT3* affect IAA and JA accumulation in the plants subjected to pathogen infection. Differently from *cat2-1*, both *cat1-1* and *cat3-1* mutants had similar IAA level to the wild-type when challenged by *Pst* DC3000, and they exhibited JA accumulation similar to the wild-type when subjected to *B. cinerea* infection (Figure S7); this suggests that *CAT1/3* may not function in changes of IAA or JA accumulation in the plants challenged by the pathogens.

In summary, our study shows that CAT2 participates in SA-mediated repression of auxin accumulation resulting from changes in Trp accumulation due to TSB1 sulfenylation mediated by higher H_2O_2 levels derived from SA inhibition of CAT2. CAT2 also functions in SA-mediated inhibition of JA biosynthesis via changes in ACX2/ACX3 activity through direct interaction with these enzymes. While this SA-mediated suppression of auxin signaling through CAT2 confers resistance to a biotrophic pathogen, the decrease in JA accumulation due to the interaction of SA/CAT2/ACX results in greater susceptibility to a necrotrophic pathogen.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

The published transgenic and mutant lines used in our research are *DR5::GUS*, *cat2*-1 (SALK_076998), *cat2-1 DR5::GUS*, and *cat2-1 CAT2::iaaM-11* (Gao et al., 2014). The lines *acx2-1* (SALK_006486), *acx3-1* (SALK_044956), and *sid2-2* (CS16438); plasmid PTS-RFP; and *Arabidopsis* cDNA library (CD4-10) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The mutants *cat2-1 sid2-2*, *acx2-1 acx3-6*, and *cat2-1 acx2-1 acx3-6* were generated by genetic cross. Sequences of primers used to identify the mutant by PCR are listed in Table S1. *Arabidopsis* plants were grown in soil at 22°C under 16/8 hr day/night cycles, and the intensity of the light was set at 150 μmol $m^{-2}s^{-1}$.

Sulfenic Acid Modification Assays

Sulfenic acid modification assay of Arabiodopsis proteins was performed as in previous reports (Kim et al., 2002; Saurin et al., 2004). Briefly, proteins were treated with H_2O_2 at various concentrations for 30 min at room temperature, incubated at 50°C for 20 min by adding four volumes of blocking buffer (20 mM methy methanethiosulfonate, MMTS; 2.5% SDS) to block the free thiols of cysteine, and then precipitated with two volumes of ice acetone. The precipitant was resuspended in HENS buffer (25 mM HEPES-NaOH [pH 7.7], 1 mM EDTA, 0.1 mM Neocuproine, 1% SDS). To label the −SOH group of protein with biotin tag, Biotin-HPDP and sodium arsenite were added into the suspension to 2 mM and 1 mM, respectively, and the suspension was incubated at room temperature for 1 hr to reduce −SOH to −SH, which can react with Biotin-HPDP. After removal of excess Biotin-HPDP with acetone, the proteins were resuspended in HENS buffer. Then two volumes of neutralization buffer (20 mM HEPES-NaOH [pH 7.7], 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100) and streptavidin-agarose were added to bind biotinylated proteins at 4°C, and the beads were washed with wash buffer (20 mM HEPES-NaOH [pH 7.7], 1 mM EDTA, 600 mM NaCl, 0.5% Triton X-100) four times. Finally, the sulfenylated proteins were visualized on nonreducing SDS-PAGE and western blots probed with streptavidin-HRP.

To further confirm TSB1 sulfenylation in vivo, total sulfenylated proteins from the leaves of 4-week-old *Arabidopsis* plants were isolated as described above. Then, the isolated total sulfenylated proteins were separated by SDS-PAGE, and sulfenylated TSB1 was detected by immunoblot with an antibody against TSB1.

Yeast Two-Hybrid Assays

To identify CAT2-interacting proteins with the yeast two-hybrid system, the CDS of *CAT2* was fused to the DNA binding domain (BD) in pGBKT7 and used to screen the pACT (containing activating domain, AD)-based *Arabidopsis* cDNA library (CD4-10, ABRC) according to the MATCHMAKER User Manual and Yeast Protocols Handbook (Clontech). A total of 1×10^6 yeast transformants were screened on SD/-His-Trp-Leu-Ade plates (Clontech).

To further verify the interaction between CAT2 and ACX2/ACX3 in yeast, the CDS of ACX2 and ACX3 were individually cloned into the pGADT7. Yeast

transformation and growth were carried out with the Matchmaker system (Clontech). Primer sequences are listed in Table S1.

Quantitative Real-Time PCR

RNA was extracted from plant leaves using TRIzol Reagent (Invitrogen). After treatment with RQ1 RNase-free DNase (Promega), first-strand cDNA synthesis was carried out using Superscript II Reverse Transcriptase according to the manufacturer's instructions (TOYOBO). The constitutively expressed *EIF4A* gene was used as an internal control. qRT-PCR analysis was performed on a Bio-RAD CFX96 system with the dye SYBR Green (Invitrogen). All experiments were repeated at least three times. Primer sequences are listed in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2017.01.007.

AUTHOR CONTRIBUTIONS

H.-M.Y., W.-C.L., and Y.-T.L. conceived and designed the experiments. H.-M.Y. and W.-C.L. performed the experiments. W.-C.L. and Y.-T.L. wrote the paper.

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