Phenylalanine Suppresses Cell Death Caused by Loss of Fumarylacetoacetate Hydrolase in *Arabidopsis*

Yihe Jiang
Hunan Agricultural University
Qi Zhu
Hunan Agricultural University
Hua Yang
Hunan Agricultural University
Tiantian Zhi
Yichun University
Chunmei Ren (rencm@hunau.net)
Hunan Agricultural University

Research Article

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Abstract

Fumarylacetoacetate hydrolase (FAH) catalyzes the final step of Tyrosine (Tyr) degradation pathway essential to animals and the deficiency of FAH causes an inborn lethal disease. In plants, a role of this pathway was unknown until we found that mutation of *Short-day Sensitive Cell Death1* (*SSCD1*), encoding *Arabidopsis* FAH, results in cell death under short day. Phenylalanine (Phe) could be converted to Tyr and then degraded in both animals and plants. Phe ingestion in animals worsens the disease caused by FAH defect. However, in this study we found that Phe represses cell death caused by FAH defect in plants. Phe treatment promoted chlorophyll biosynthesis and suppressed the up-regulation of reactive oxygen species marker genes in the *sscd1* mutant. Furthermore, the repression of *sscd1* cell death by Phe could be reduced by α-aminooxi-β-phenylpropionic acid but increased by methyl jasmonate, which inhibits or activates Phe ammonia-lyase catalyzing the first step of phenylpropanoid pathway, respectively. In addition, we found that jasmonate signaling up-regulates *Phe ammonia-lyase 1* and mediates the methyl jasmonate enhanced repression of *sscd1* cell death by Phe. These results uncovered the relation between chlorophyll biosynthesis, phenylpropanoid pathway and jasmonate signaling in regulating the cell death resulting from loss of FAH in plants.

Introduction

Tyrosine (Tyr) degradation pathway includes the five-step enzymatic reactions, in which Tyr is first converted to 4-hydroxyphenylpyruvate by Tyr aminotransferase, then transformed into homogentisate by 4-hydroxyphenylpyruvate dioxygenase, next, homogentisate is catalyzed by homogentisate dioxygenase (HGO) to form maleylacetoacetate (MAA) that is isomerized by MAA isomerase (MAAI) to fumarylacetoacetate (FAA) and last hydrolyzed by fumarylacetoacetate hydrolase (FAH) to fumarate and acetoacetate[1,2]. Tyr degradation is essential to animals, blockage of this pathway results in metabolic disorder diseases, in which the most severe disorder in humans is hereditary tyrosinemia type I (HT1), an inborn lethal disease caused by deficiency of FAH[3,4]. Loss of FAH in HT1 patients results in the accumulation of Tyr degradation intermediates including FAA and MAA, and then both would undergo spontaneous reduction to succinylacetoacetate followed by spontaneous nonenzymatic decarboxylation to succinylacetone (SUAC), which is toxic to cells and tissues[3]. Although the homologous genes putatively encoding HGO, MAAI, and FAH were demonstrated to exist in plants[2,5], a role of Tyr degradation pathway in plants had been unclear until we cloned the *short-day sensitive cell death 1* (*SSCD1*) gene encoding an Arabidopsis putative FAH[6]. Loss of FAH in the *sscd1* mutant leads to spontaneous cell death under short-day conditions (SD)[6] and the accumulation of SUAC[7]. SUAC inhibits an activity of δ-aminolevulinic acid dehydratase (ALAD) involved in Chlorophyll (Chl) biosynthesis, resulting in high production of protochlorophyllide (Pchlide), an intermediate of Chl biosynthesis, in the dark under SD and excessive accumulation of Pchlide induces the production of reactive oxygen species (ROS) upon light irradiation and thereby causes cell death[8].

Phenylalanine (Phe) is catalyzed by Phe hydroxylase to form Tyr and then degraded in animals, and dietary restriction of Tyr and Phe can improve the condition of HT1 patients[4,9]. In plants, Phe could be also converted into Tyr and then via homogentisate, to plastoquinones and tocopherols or to degradation of the aromatic ring[10], however, it can carry through phenylpropanoid metabolism to produce secondary metabolites[10]. Phenylpropanoids are precursors to flavonoids, isoflavonoids, cumarins, and stilbenes, which have important functions in plant defense against pathogens and other predators, as UV light protectants, and as regulatory molecules in signal transduction and communication with other organisms[11]. In phenylpropanoid pathway, the first step is that Phe ammonia-lyase (PAL) catalyzes the deamination of Phe to give cinnamic acid[12,13]. The α-aminooxi-β-phenylpropionic acid (AOPP) is an inhibitor of PAL, treatment with AOPP could reduce the activity of PAL[14–16]. Jasmonates (JAs) including jasmonic acid, methyl jasmonate (MeJA), and other derivatives, are a basic class of plant hormones involved in plant growth, development, and responses to biotic and abiotic stresses[17–20]. Treatment with MeJA could increase the activity of PAL in plants such as Chinese bayberry[21], wheat[22] and tuberose[16]. The *PAL* gene expression is responsive to a variety of environmental stimuli, including pathogen infection, wounding, nutrient depletion, UV irradiation, extreme temperatures, and other stress conditions[23–26].

To investigate whether the uptake of Phe in plants increases the cell death caused by loss of FAH like as that in animals, in this study, the *sscd1* mutant was treated with Phe and it was found that the death of *sscd1* seedlings was not increased but suppressed by Phe treatment. With further investigation, we found that Phe treatment promoted Chl biosynthesis and repressed the upregulation of ROS marker genes in the *sscd1* mutant. Furthermore, AOPP treatment reduced the repression of *sscd1* cell death by Phe. In addition, MeJA treatment enhanced the repression of *sscd1* cell death by Phe as well as upregulated the *PAL1* gene, both of which are mediated by JA signaling. Our work suggested that the effects of Phe on the cell death resulting from loss of FAH are different in plants and animals and uncovered the relation between Chl biosynthesis, phenylpropanoid pathway and JA signaling in regulating the cell death resulting from loss of FAH, which will help to further investigate the regulation of Tyr degradation, phenylpropanoid biosynthesis and the cell death in plants.

Results

Phe treatment suppresses the death of sscd1 seedlings

Since Phe could be converted into Tyr and then degraded in plants[10], we wondered whether Phe treatment promotes the death of *sscd1* seedlings. To this end, the seeds of wild type and the *sscd1* mutant were plated on MS medium without or with 0.1, 0.5, 1 and 2 mM Phe and grown under SD. Unexpectedly, the death of *sscd1* seedlings treated with Phe was not increased, on the contrary, it was reduced (Fig. 1). When medium was supplemented with 0.1mM Phe, the death rate of *sscd1* seedlings was slightly reduced compared to that without Phe, however, it was significantly reduced as concentrations of Phe was increased (Fig. 1a). For example, on the 7th day, more than 80% of *sscd1* seedlings in medium

without Phe were dead, however, when medium was supplemented with 0.5, 1 and 2 mM Phe, the rates of death seedlings were only about 70%, 30%, and 5%, respectively (Fig. 1a). The phenotype of seedlings shown in Fig. 1b clearly displayed that Phe treatment reduced the death of *sscd1* seedlings. Similarly, when *sscd1* seedlings grown in medium with Phe under LD were transferred to SD, the extent of cell death was obviously attenuated (Fig. 1c). All these results indicated that Phe treatment suppresses the death of *sscd1* seedlings.

The upregulation of ROS marker genes in sscd1 could be repressed by Phe treatment

Previously, we have speculated that the production of ROS resulting from excessive accumulation of Pchlide causes the *sscd1* cell death[8]. Because ROS marker genes such as *ascorbate peroxidase 2 (APX2)*[27], *oxidative signal inducible 1 (OXI1)*[28,29], *bonzai1-associated protein 1 (BAP1)* and *transcription factor (ZP)*[30] were up-regulated before an occurrence of cell death in the *sscd1* mutant[8,31], we next investigated whether the repression of *sscd1* cell death by Phe treatment is correlated with expression levels of these genes. The cell death of *sscd1* seedlings which were transferred from LD to SD occurred on the 4th day[6], so, the seedling transferred from LD to SD for 3 days growth was collected and expression levels of genes were assessed by an analysis of real-time quantitative PCR (RT-qPCR). As shown in Fig. 2, the expression levels of *APX2*, *OXI1*, *BAP1* and *ZP* in the *sscd1* mutant was significantly increased compared to that in wild type, however, they were clearly reduced after Phe treatment. Therefore, the up-regulation of ROS marker genes in *sscd1* could be repressed by Phe treatment. Since the expression levels of ROS marker genes is positively correlated to the content of ROS[27,29,30], the repression of up-regulation of ROS marker genes in *sscd1* by Phe treatment (Fig. 2) indicated less production of ROS after Phe treatment.

Chl biosynthesis could be promoted by Phe treatment

Since the *sscd1* cell death is mediated by Chl biosynthetic pathway[8], we next investigated whether treatment of Phe influences Chl biosynthesis. We first determined the content of Chl and found that it was increased after Phe treatment (Fig. 3a). Since *HEMA1* and *CHLH* are two pivotal genes regulating Chl biosynthesis[32], we next tested the expression of these genes by RT-qPCR. As we expected, the expression levels of *HEMA1* and *CHLH* in both wild type and *sscd1* were also increased after Phe treatment (Fig. 3b, c). Therefore, Phe treatment promotes Chl biosynthesis.

Repression of the sscd1 cell death by Phe could be reduced by AOPP and enhanced by MeJA

Phe is a precursor of phenylpropanoid biosynthesis and PAL catalyzes the first step of this pathway[33]. If the repression of *sscd1* cell death by Phe is related to phenylpropanoid biosynthesis, it would be changed by an inhibition or activation of PAL. To conform that, firstly, the *sscd1* seedlings were treated with AOPP, a potent inhibitor of PAL[15], on the basis of Phe treatment. As we expected, after treated with AOPP the death seedlings were clearly increased (Fig. 4a). For example, the death rate of 7-d-old *sscd1* seedlings treated with 1 mM Phe was approximately 30%, however, when seedlings were treated with 1 mM Phe

and 100 μM AOPP, it was increased to approximately 72% (Fig. 4b). These results indicated that the inhibition of PAL activity by AOPP could reduce repression of the *sscd1* cell death by Phe.

Since the activity of PAL could be activated by MeJA[16], we next investigated whether treatment with MeJA enhances the repression of *sscd1* cell death by Phe. As shown in Fig. 5a, in the absence of Phe, treatment with 5 μM MeJA did not distinctly affect the phenotype of both wild type and *sscd1* seedlings, however, when treated with 5 μM MeJA and 0.5 mM Phe the green seedlings of *sscd1* obviously increased compared to those only treated with 0.5 mM Phe. The death rate of 7-d-old *sscd1* seedlings treated with 0.5 mM Phe was approximately 64% whereas it was less than 40% once treated with 5 μM MeJA and 0.5 mM Phe (Fig. 5b). Therefore, the activation of PAL activity by MeJA could enhance repression of the *sscd1* cell death by Phe.

Treatment with MeJA causes the COI1 dependent upregulation of PAL1

In Arabidopsis, PAL is encoded by a small gene family including *PAL1*, *PAL2*, *PAL3*, and *PAL4*[26,34]. We next investigated whether treatment with MeJA affects some or all of these genes' expression, and if do, is it dependent on COI1[17], a JA receptor of JA signaling[35]? To this end, the seedlings of wild type and the *coi1-2* mutant were treated with MeJA and the expression levels of *PAL1*, *PAL2*, *PAL3*, and *PAL4* were assessed by an analysis of RT-qPCR. As shown in Fig. 6, after treatment of MeJA, the expression level of *PAL1* was significantly increased in wild type but not in *coi1-2*. However, the expression levels of *PAL2*, *PAL3*, and *PAL4* were not significantly altered in both wild type and *coi1-2* after treated with MeJA (Fig. 6). These results suggested that treatment with MeJA upregulates *PAL1*, and this up-regulation is dependent on *COI1.*

Mutation of COI1 could eliminate MeJA-increased Phe repression of sscd1 cell death

Since treatment with MeJA enhanced the repression of *sscd1* cell death by Phe (Fig. 5) as well as up-regulated *PAL1* in dependence of *COI1* (Fig. 6), we next investigated whether mutation of *COI1* alters the effect of MeJA on Phe in inhibiting the *sscd1* cell death. The seeds of the *sscd1* single mutant and the *sscd1coi1* double mutant were plated on medium added without or with 0.5 mM Phe or/and 5 μM MeJA and grown under SD, and then the death seedlings were counted. As shown in Fig. 7a, the death rate of *sscd1coi1* seedlings was lower than that of *sscd1*, which is consistent with our previous study[31]. Phe treatment also significantly reduced the death seedlings of *sscd1coi1* (Fig. 7a, right). However, treated with MeJA and Phe, not as in *sscd1* (Fig. 7a, left), the death seedlings of *sscd1coi1* were not significantly reduced compared to that treated with Phe (Fig. 7a, right). The phenotype of *sscd1* and *sscd1coi1* seedlings in Fig. 7b clearly displayed that MeJA treatment enhances the repression of seedlings death by Phe in *sscd1* but not in *sscd1coi1* (Fig. 7b, right). Therefore, mutation of *COI1* could eliminate the MeJA-increased Phe repression of *sscd1* cell death.

Discussion

FAH catalyzes the final step of Tyr degradation pathway and the deficiency of FAH in animals causes an inborn lethal disease, which was named as HT1 in humans[1,3]. Phe could be converted to Tyr and then degraded in animals, the dietary restriction of Tyr as well as Phe can improve the condition of HT1 patients[4,9]. In plants, the *SSCD1* gene encodes the Arabidopsis FAH, mutation of *SSCD1* results in spontaneous cell death under SD[6]. Like as in animals, Phe could be also converted into Tyr in plants and then degraded[10]. However, in our study the death of *sscd1* seedlings was not increased but repressed by Phe treatment (Fig. 1). So, why would Phe treatment repress the cell death resulting from loss of FAH in plants?

Previously, we demonstrated that the *sscd1* cell death is mediated by Chl biosynthesis[8]. The inhibition of the ALAD activity by SUAC in the *sscd1* mutant influences Chl biosynthesis resulting in impairment of feedback inhibition of Chl biosynthesis from the light–dark transition under SD, which activates Chl biosynthesis and accumulation of Pchlide in the dark, and then upon re-illumination the excessive accumulation of Pchlide induces the mass production of ROS and thereby causes cell death[8]. Thus, it is the excess ROS that induces the *sscd1* cell death. In this study, treatment of *sscd1* seedlings with Phe distinctly repressed the up-regulation of ROS marker genes including *APX2*, *OXI1*, *ZP* and *BAP1* (Fig. 2), which indicated that ROS is reduced by Phe treatment.

In Chl biosynthetic pathway there are two pivotal control points mainly regulated by *HEMA1* and *CHLH* respectively[32,36–38]. In our study, after treatment with Phe, both the content of Chl and the expression levels of *HEMA1* and *CHLH* were increased (Fig. 3), suggesting that Phe treatment could promote Chl biosynthesis. The increase in Chl biosynthesis would restore to some extent the feedback inhibition of Chl biosynthesis in the *sscd1* mutant from the light–dark transition, as a result, reducing the accumulation of Pchlide in the dark and the production of ROS after subsequent exposure to light.

In plants, Phe could be metabolized through the phenylpropanoid pathway to produce secondary metabolites, which plays an important role in plant against stress including UV-light, drought and pathogen attack[10,11,26,39–41]. PAL catalyzes the first step of the phenylpropanoid pathway, which is a key step in phenylpropanoid biosynthesis[13,34,42]. The activity of PAL could be inhibited by AOPP and promoted by MeJA[16]. Treatment with AOPP prevents the increase in resistance to *B. cinerea* due to the application of external Phe[41]. In our study, the repression of *sscd1* seedlings death by Phe was reduced by AOPP treatment (Fig. 4), however, it was enhanced by MeJA (Fig. 5), which suggested that the suppression of *sscd1* cell death by Phe is related to the phenylpropanoid pathway. Since the secondary metabolites produced by Phe metabolism through the phenylpropanoid pathway have antioxidant function[41], ROS could be also reduced by the metabolism of Phe through the phenylpropanoid pathway, which should be another important cause for the repression of *sscd1* cell death by Phe.

Recently, we found that JA signaling is involved in the *sscd1* cell death[31]. In the *sscd1* mutant, the accumulation of SUAC results in the generation of ROS, which induces cell death as well as JA synthesis[31]. JA up-regulates Tyr degradation pathway, producing more SUAC, which promotes cell

death[31]. Once JA signaling is broken by mutation of *COI1* encoding a JA receptor[35], the up-regulation of Tyr degradation pathway by JA is eliminated, reducing the production of SUAC, as a result, the *sscd1* cell death is repressed[31]. In this study, MeJA treatment markedly increased the expression level of *PAL1* in wild type but not in the *coi1-2* mutant (Fig. 6), indicating that JA signaling up-regulates the phenylpropanoid pathway. The repression of *sscd1* cell death by Phe could be enhanced by MeJA treatment in the *sscd1* single mutant (Figs. 5 and 7) but not in the *sscd1coi1* double mutant (Fig. 7), which suggested that MeJA treatment enhances Phe inhibition of the *sscd1* cell death through JA signaling. Therefore, JA has a dual regulatory effect on the *sscd1* cell death. On the one hand, JA up-regulates the Tyr degradation pathway, promoting the *sscd1* cell death, on the other hand, JA up-regulates the phenylpropanoid pathway, inhibiting the *sscd1* cell death. For this reason, the death of *sscd1* seedlings was not increased by MeJA treatment, it seemed to decrease slightly (Figs. 5 and 7), which suggested that the effect of MeJA treatment on the *sscd1* cell death through phenylpropanoid pathway might be greater than that through Tyr degradation pathway.

In conclusion, although Phe can be degraded through Tyr degradation pathway, unlike in animals, Phe treatment does not increase the cell death resulting from loss of FAH in plants, instead, it represses the cell death. A possible mechanism for the repression of *sscd1* cell death by Phe treatment can be described as follows (Fig. 8). Loss of FAH in the *sscd1* mutant results in a decline of Chl biosynthesis, which impairs the feedback inhibition of Chl biosynthesis from light–dark transition under SD, leading to the accumulation of ROS and then cell death. Phe treatment, on the one hand, promotes Chl biosynthesis, increasing the feedback inhibition of Chl biosynthesis from light–dark transition under SD, and on the other hand, activates phenylpropanoid pathway, both of which reduce ROS and subsequent cell death. In addition, in the *sscd1* mutant ROS induces cell death as well as JA synthesis. JA signaling up-regulates Tyr degradation pathway, promoting the *sscd1* cell death, however, it also up-regulates *PAL1* that activates phenylpropanoid pathway, repressing the *sscd1* cell death. Since the effect of MeJA treatment on the *sscd1* cell death through phenylpropanoid pathway might be greater than that through Tyr degradation pathway, the repression of *sscd1* cell death by Phe could be enhanced by MeJA treatment.

Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana **L. ecotype Columbia-0 (Col-0) was obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH, USA) and the mutants used in this study are in Col-0 background. The *sscd1* mutant[6] was isolated by Han et al (2013) in our laboratory. The *coi1-2* mutant[43] was kindly provided by Professor Xie (Tsinghua University, Beijing, China) and the *sscd1coi1* double mutant[31] was generated through a cross of *sscd1* with *coi1-2* by Zhou et al (2020) in our laboratory. Experimental research on plants including the collection of plant material was performed in accordance with relevant institutional, national, and international guidelines and legislation.**

Seeds were surface sterilized with 20% (v/v) chlorine bleach containing 0.1% (v/v) Triton X-100 for 10 min and washed three to five times with sterile water, then plated on Murashige & Skoog medium supplemented with 1% (m/v) sucrose and 0.7% (w/v) agar (pH 5.8) (MS). The different concentrations of Phe (SIGMA) were added in MS medium. Plates were chilled at 4 °C in darkness for 3 days and then transferred to a growth chamber with LD (16-h light/8-h dark) or SD (8-h light/16-h dark) under 150 μmol photons m^{-2} s^{-1}, controlled temperature (22±2 °C).

For RT-qPCR analysis (Figs. 2, 3b) and determination of Chl content (Fig. 3a), One-week-old seedlings growing under LD were transplanted onto MS added without or with Phe and grown under LD for an additional 1 week's growth, and then transferred to SD for three days growth. The seedlings were harvested at the end of the third day's light for the determination of Chl content or harvested at 2 hours light after three days for RT-qPCR analysis.

Determination of the dead seedlings

A dead seedling is one for which all leaves were completely bleached. The rate of seedling death was calculated as the percentage of dead seedlings. The total number of seedlings counted was approximately 100, and the experiment was performed in three independent biological repeats.

RT-qPCR analysis

Total RNA was isolated using TRIZOL reagent (Life Technologies, https://www.thermofisher.com/us/en/home/brands/life-technologies.html). After incubation with DNase I (RNase Free, Thermo Fisher Scientific, https://www.thermofisher.com/) at 37°C for 30 min and then at 65°C for 10 min to remove genomic DNA, the RNA concentration and purity were measured spectrophotometrically using OD260/OD280 and OD260/OD230 ratios (ND-1000, NanoDrop, THERMO FISHER SCIENTIFIC). Complementary DNA was synthesized from the mixture of oligo-dT primers and random primers using a ReverTraAce qPCR RT kit (perfect real time) according to the manufacturer's instructions (Toyobo, http://www.toyobo-global.com/).

RT-qPCR was performed in 96-well blocks using a SYBR qPCR mix (Roche, https://lifescience.roche.com/) with a Bio-Rad CFX Connect™ Real-Time PCR detection system (http://www.biorad.com/) following the manufacturer's instructions. The RT-qPCR amplifications were performed under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The primers of genes tested by RT-qPCR are listed in Table 1, and *ACTIN2* was used as an internal control. The gene expression for each sample was calculated on three analytical replicates, and the relative expression was quantified using the $2^{-\triangle\triangle Ct}$ method. The experiment was performed in three independent biological repeats. The significance of differences between datasets was evaluated using the two-tailed Student's t-test.

Determination of Chl content

The content of Chl was determined referring to the method described by Lichtenthaler[44] (1987). Weighed segments of frozen crushed material (about 0.04 g) were homogenized in 1 mL 80% acetone and stood for 5 to 6 hours, then centrifuged for 10 min at 5000 rpm at 4°C and assayed spectrophotometrically at 663 nm and 645 nm. Result calculation: $C_{(mg/g)} = (17.32\ A_{663}+7.18\ A_{645})/m_{(g)}$. The experiment was performed in three independent biological repeats.

AOPP treatment

Seeds were germinated on MS added with 1 mM Phe (SIGMA) and grown under SD. On the third day seedlings were sprayed with 100 μM AOPP (Wako) or ddH_2O (as a control) once a day for 5 days, then the rate of death seedlings was counted, and the seedlings were photographed. The experiment was performed in three independent biological repeats.

MeJA treatment

For determination of dead seedlings (Figs. 5 and 7), seeds were germinated on MS added without or with 0.5 mM Phe and/or 5 μM MeJA (SIGMA) and grown under SD for 6-8 days. For RT-qPCR analysis (Fig. 6), about two-week-old seedlings growing under LD were transferred to SD and on the fourth day the seedlings were sprayed with 100 μM MeJA or ddH_2O (as a control). After MeJA treatment for one day the seedlings were harvested and used for RT-qPCR analysis. The experiment was performed in three independent biological repeats.

Declarations

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Author contribution statement

C.R. conceived, designed and supervised the experiments; Y.J. and T.Z. performed the experiments; Q.Z. and H.Y. provided technical assistance to Y.J.; Y.J., Q.Z. and C.R. analyzed the data; Y.J. wrote the article with contributions from all authors; and C.R. supervised, modified and complemented the writing.

Competing interests

The authors declare no competing interests.

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Table

Table 1 Primers of genes tested by real-time quantitative PCRs

Gene	Forward primer	Reverse primer
APX2 (AT3G09640)	5′-ACAAAGTTGAGCCACCTCCT-3′	5′-AAGGTGTGTCCACCAGACAA-3′
OXI1 (AT3G25250)	5′-GTTGAGGAAATCAAGGGTCATG-3′	5′-TGGACGATATTCTCCACATCC-3′
ZP (AT5G04340)	5′-TACGAAGGAAAGAACGGAGGC-3′	5′-GGTATCGGCGGTATGTTGAGG-3′
CHLH (AT5G13630)	5′-CAGCCAACATCAGTCTTGCT-3′	5′-ACCTGCTTCTTCTCAGCCAT-3′
BAP1 (AT3G61190)	5′-ATCGGATCCCACCAGAGATTACGG-3′	5′-AATCTCGGCCTCCACAAACCAG-3′
HEMA1 (AT1G58290)	5′-GTTGCTGCCAACAAAGAAGA-3′	5′-AATCCCTCCATGCTTCAAAC-3′
PAL1 (AT2G37040)	5′-TTTTGGTGCTACTTCTCATCG-3′	5′-CTTGTTTCTTTCGTGCTTCC-3′
PAL2 (AT2G37040)	5′-GTGCTACTTCTCACCGGAGA-3′	5′-TATTCCGGCGTTCAAAAATC-3′
PAL3 (AT5G04230)	5′-CAACCAAACGCAACAGCA-3′	5′-CTCCAGGTGGCTCCCTTTTA-3′
PAL4 (AT3G10340)	5'-GGTGCACTTCAAAATGAGCT-3'	5'-CAACGTGTGTGACGTGTCC-3′
ACTIN2 (AT3G18780)	5'-AGCACTTGCACCAAGCAGCATG-3'	5'-ACGATTCCTGGACCTGCCTCATC-3'

Figures

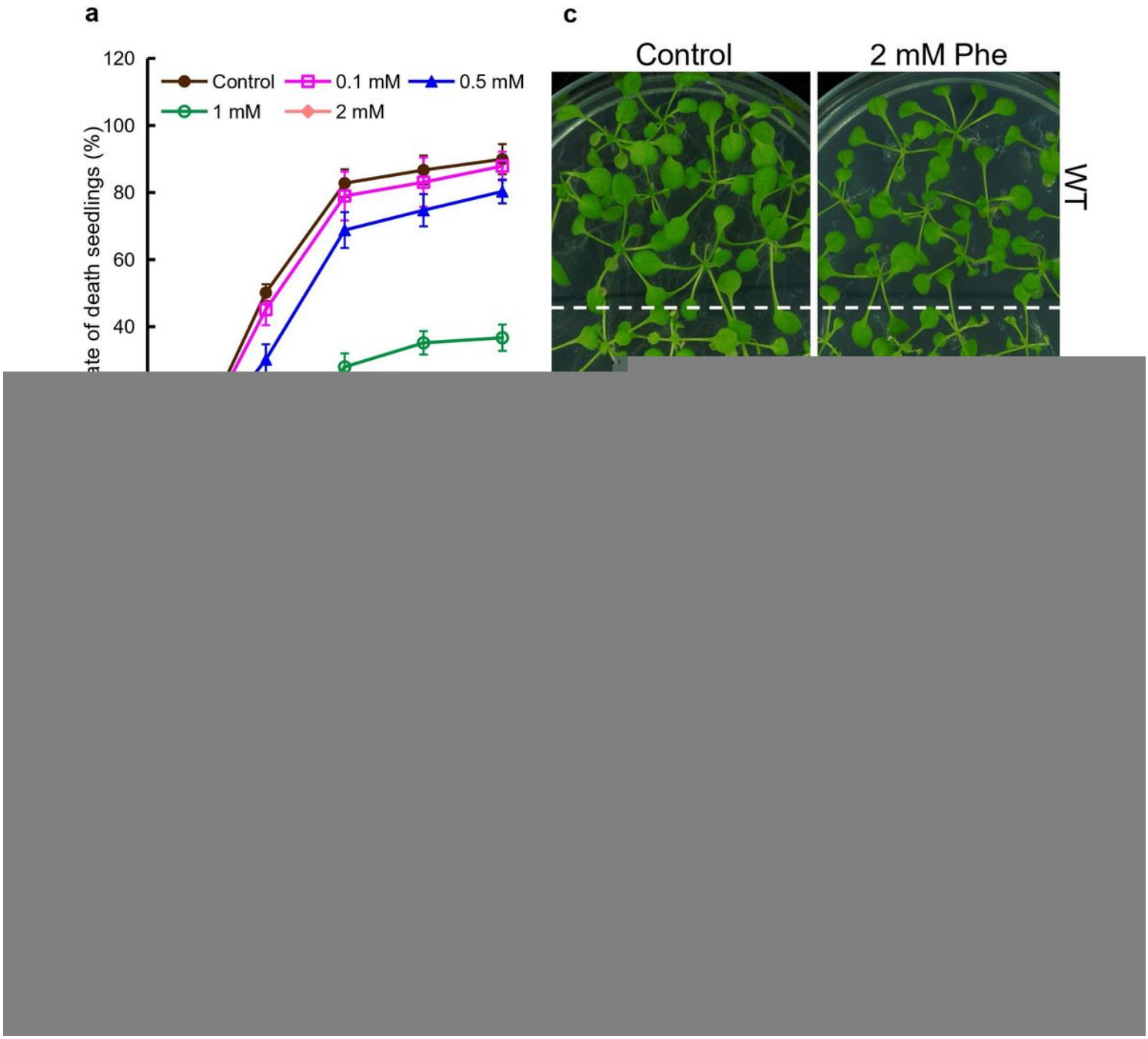

Figure 1

The death of *sscd1* seedlings was suppressed by treatment with Phe.

(**a**) The death rate of *sscd1* seedlings which were grown in medium added without (control) or with 0.1, 0.5, 1 or 2 mM Phe under SD for 5-9 days. Error bars represent standard deviations (n>30). The experiment was repeated three times. (**b**) The phenotype of WT and *sscd1* seedlings which were grown in medium added without (control) or with 0.5, 1 or 2 mM Phe under SD for 7 days. (**c**) The phenotype of WT and *sscd1* seedlings which were first grown in MS medium under LD for 7 days and transplanted to MS

medium added without (control) or with 2 mM Phe for an additional 7-d growth under LD, and then transferred to SD for a 7-d growth. Phe, Phenylalanine; SD, short day; WT, wild type, Col-0; LD, long day.

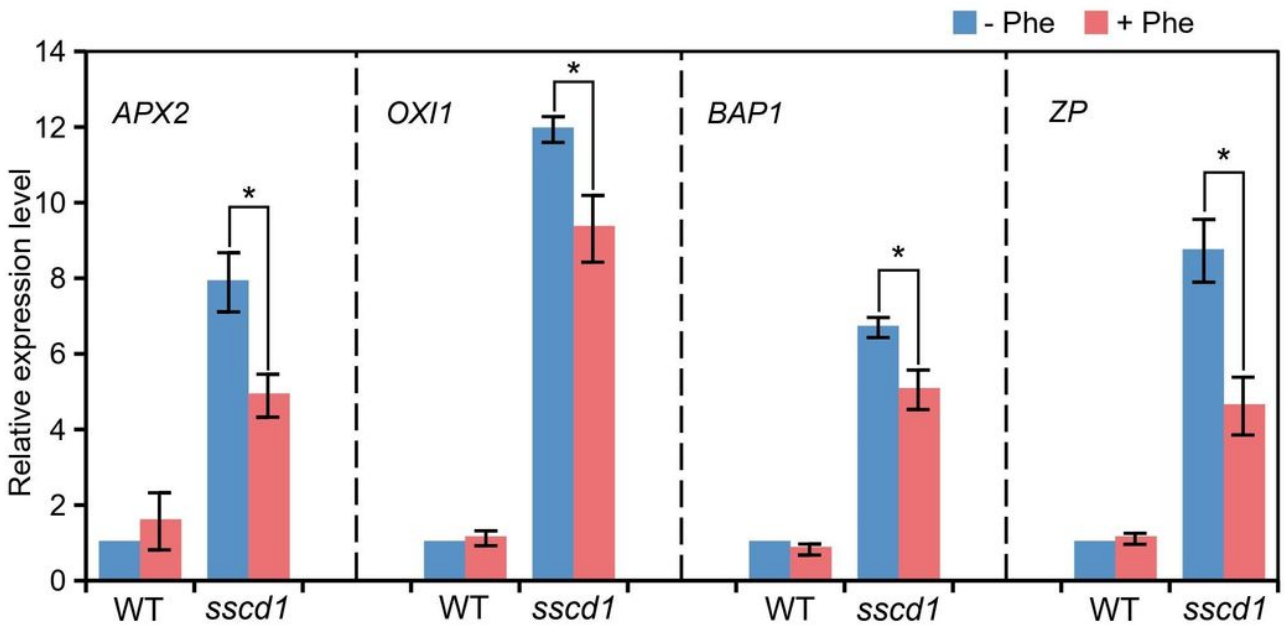

Figure 2

Expression of ROS marker genes in *sscd1* was down-regulated by treatment with Phe.

Relative expression levels of *APX2, OXI1, BAP1* and *ZP* in WT and *sscd1* seedlings which were first grown in MS medium under LD for 7 days and transplanted to MS medium added without (-) or with (+) 2 mM Phe under LD for an additional 7-d growth, and then transferred to SD for a 3-d growth. *ACTIN2* expression was used as the internal control. Each value is the mean of three biological replicates ± standard deviation. An asterisk represents the significance of differences (two-tailed Student's t-test) at the level of $P<0.05$. WT, wild type, Col-0; Phe, phenylalanine; LD, long day; SD, short day.

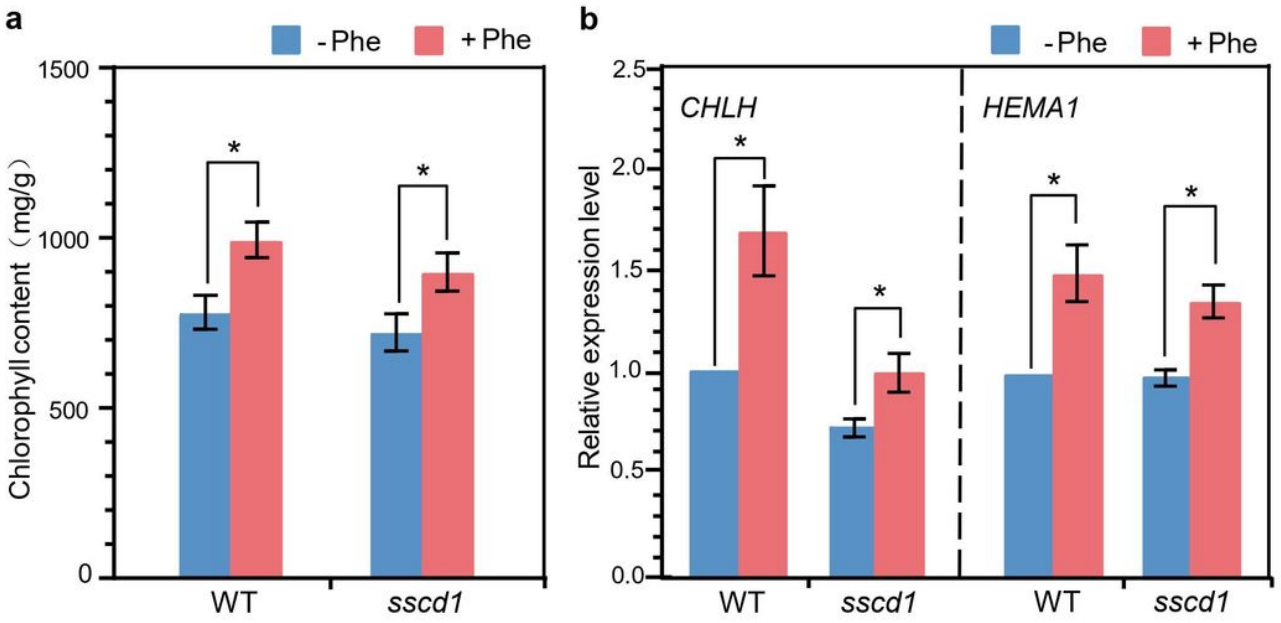

Figure 3

Chlorophyll synthesis was increased after Phe treatment.

(**a**) The content of chlorophyll in WT and *sscd1* seedlings which were first grown in MS medium under LD for 7 days and transplanted to MS medium added without (-) or with (+) 2 mM Phe under LD for an additional 7-d growth, and then transferred to SD for a 3-d growth. (**b**) The relative expression levels of *CHLH* and *HEMA1* in WT and *sscd1* seedlings which were first grown in MS medium under LD for 7 days and transplanted to MS medium added without (-) or with (+) 2 mM Phe under LD for an additional 7-d growth, and then transferred to SD for a 3-d growth. *ACTIN2* expression was used as the internal control. Each value is the mean of three biological replicates ± standard deviation. An asterisk represents the significance of differences (two-tailed Student's t-test) at the levels of $P<0.05$. WT, wild type, Col-0; LD, long day; Phe, phenylalanine; SD, short day.

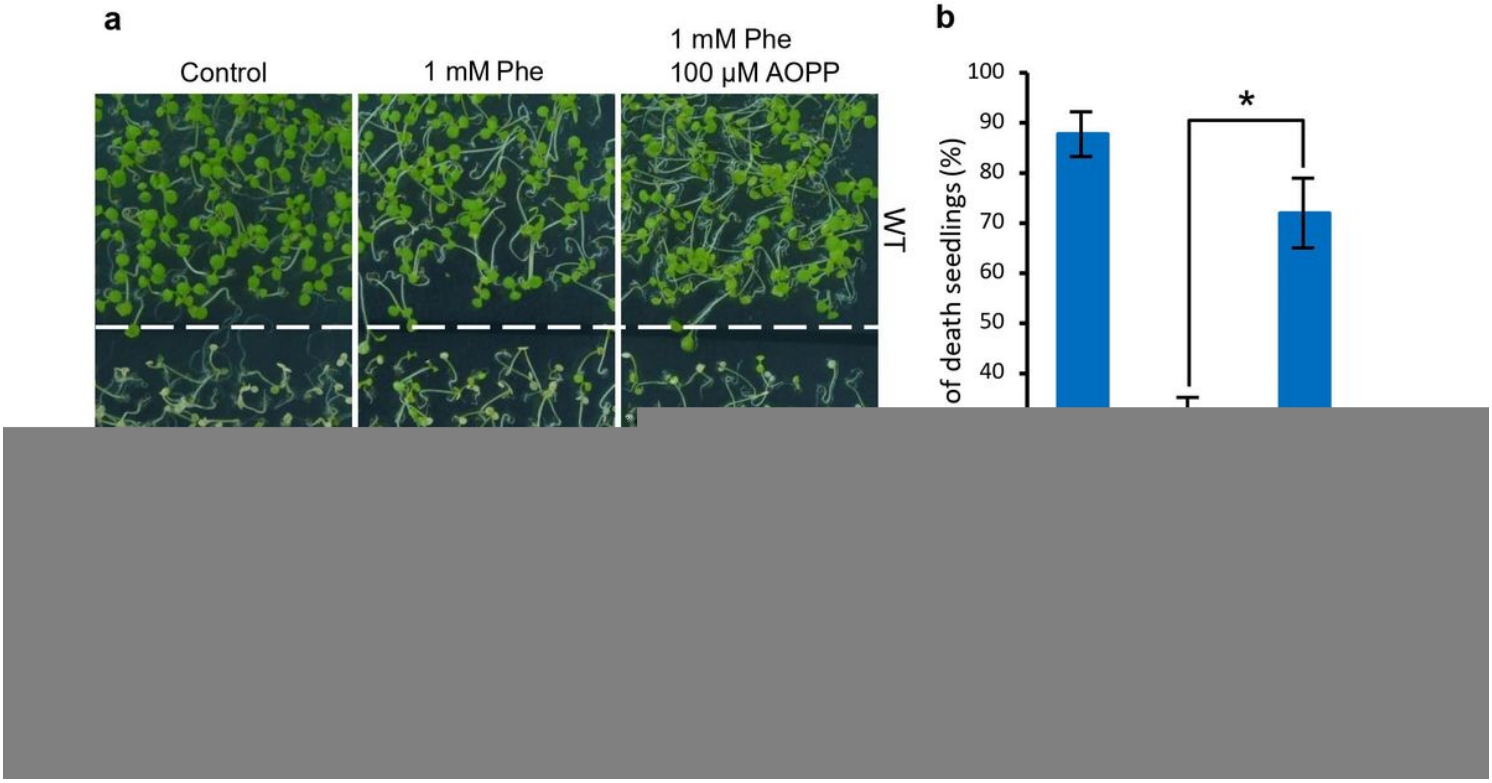

Figure 4

Treatment with AOPP reduced the inhibitory effect of Phe on the *sscd1* seedlings death.

(**a**) The phenotype of 7-d-old wild-type WT and *sscd1* seedlings which were first grown in medium added without (Control) or with 1 mM Phe under SD for 2 days and then treated without or with 100 μM AOPP for an additional 5-d growth. (**b**) The death rate of 7-d-old *sscd1* seedlings which were first grown in medium added without (Control) or with 1 mM Phe under SD for 2 days and then treated without or with 100 μM AOPP for an additional 5-d growth. Error bars represent standard deviations (n>30). The experiment was repeated three times. An asterisk represents the significance of differences (two-tailed Student's t-test) at the levels of $P<0.05$. WT, wild type, Col-0; Phe, phenylalanine; SD, short day.

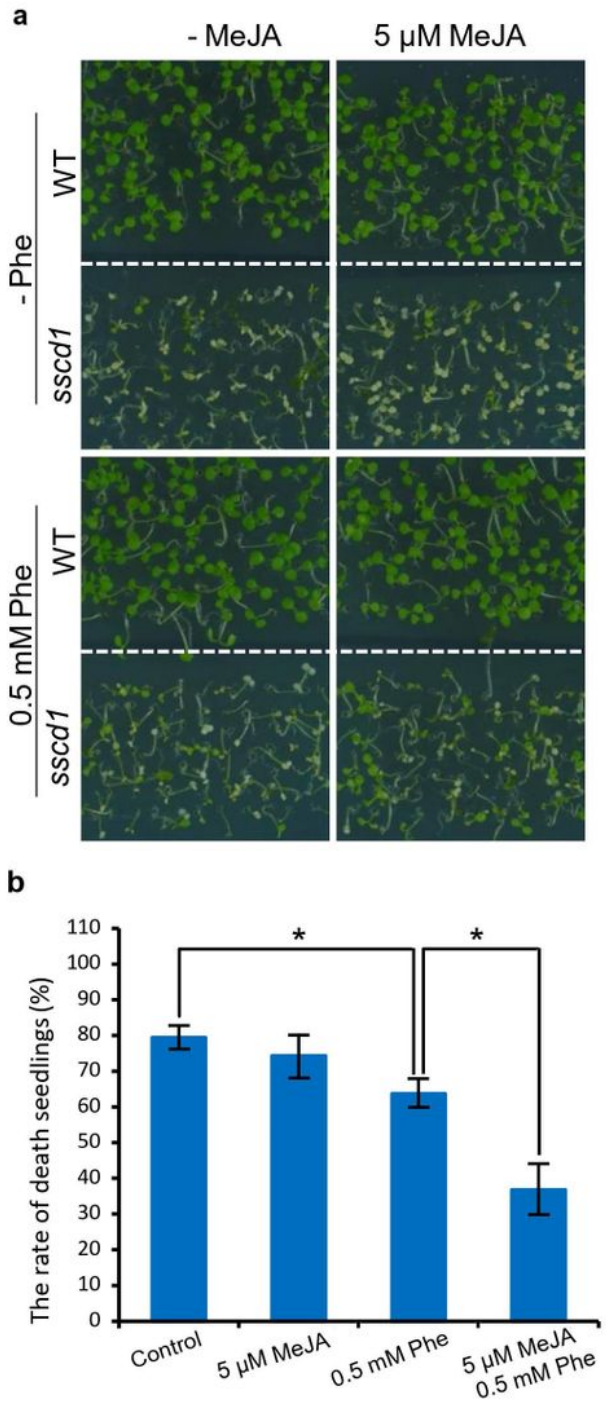

Figure 5

Treatment with MeJA enhanced the inhibitory effect of Phe on the *sscd1* seedlings death.

(**a**) The phenotype of WT and *sscd1* seedlings which were grown in medium added without (-) or with 0.5 mM Phe or/and 5 μM MeJA under SD for 7 days. (**b**) The death rate of *sscd1* seedlings which were grown in medium added without (control) or with 5 μM MeJA or/and 0.5 mM Phe under SD for 7 days. Error bars

represent standard deviations (n>30). The experiment was repeated three times. An asterisk represents the significance of differences (two-tailed Student's t-test) at the level of P<0.05. WT, wild type, Col-0; Phe, phenylalanine; MeJA, methyl jasmonate; SD, short day.

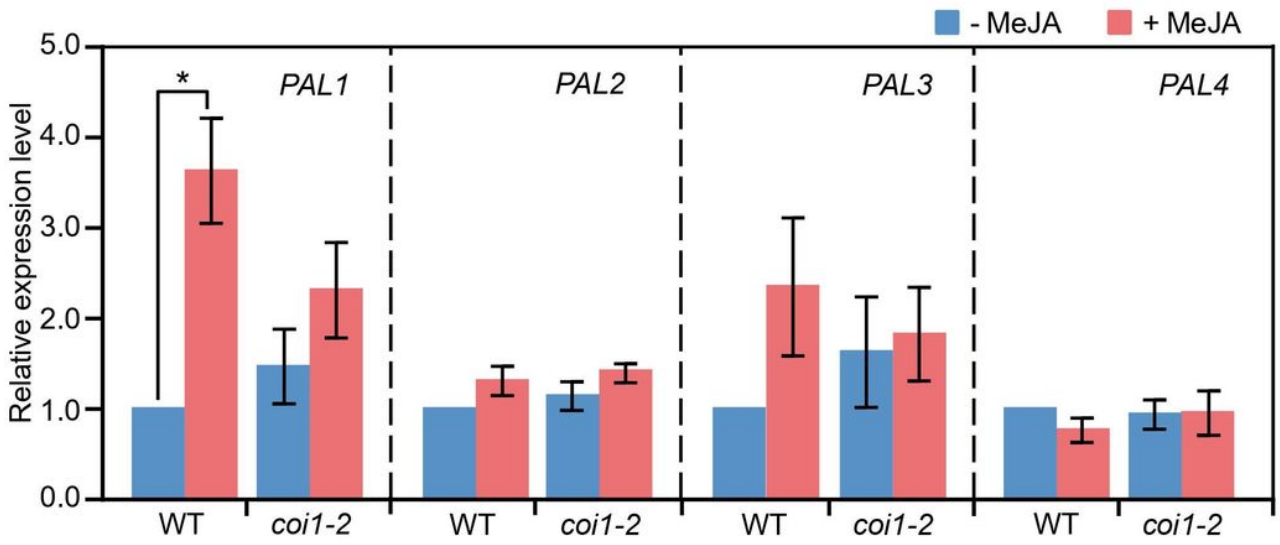

Figure 6

An analysis of expression of the *PAL* genes upon MeJA treatment by RT-qPCR.

The relative expression levels of *PAL1, PAL2, PAL3* and *PAL4* in WT and *coil-2* seedlings which were first grown in medium under LD for 12 days and removed to SD for a 3-d growth, and then treated with ddH_2O (-) or 100 μM MeJA (+) for one day. *ACTIN2* expression was used as the internal control. Each value is the mean of three biological replicates ± standard deviation. An asterisk represents the significance of differences (two-tailed Student's t-test) at the levels of P<0.05. WT, wild type, Col-0; MeJA, methyl jasmonate; LD, long day; SD, short day.

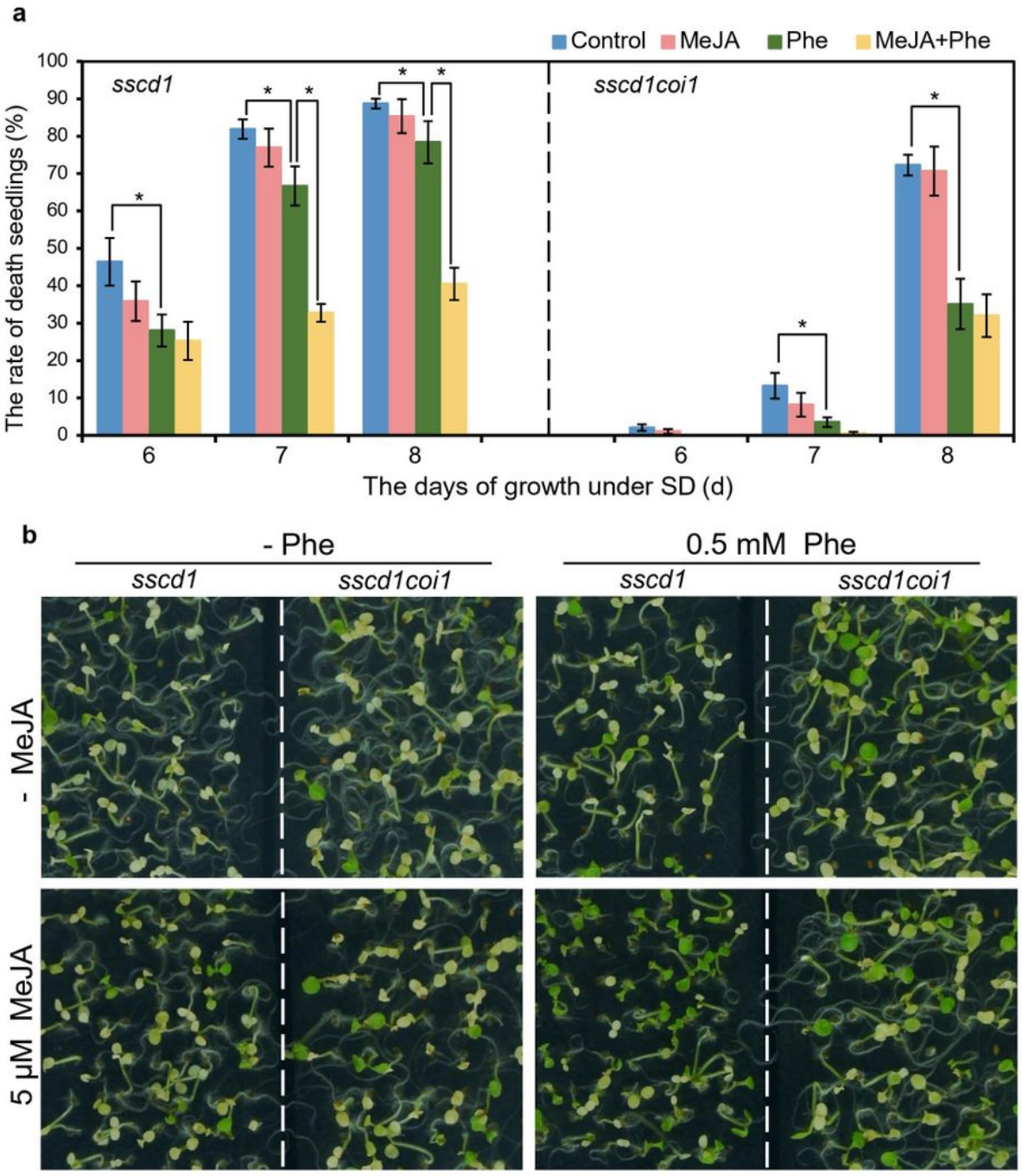

Figure 7

MeJA-increased Phe inhibition of *sscd1* seedlings death was dependent on *COI1*.

(**a**) The death rate of *sscd1* (left) and *sscd1coi1* (right) seedlings which were grown in medium added without (Control) or with 5 μM MeJA or/and 0.5 mM Phe under SD for 6-8 days. Error bars represent standard deviations (n>30). The experiment was repeated three times. An asterisk represents the

significance of differences (two-tailed Student's t-test) at the level of P<0.05. (**b**) The phenotype of *sscd1* and *sscd1coi1* seedlings which were grown in medium added without (-) or with 0.5 mM Phe or/and 5 μM MeJA under SD for 8 days. MeJA, methyl jasmonate; Phe, Phenylalanine; SD, short day.

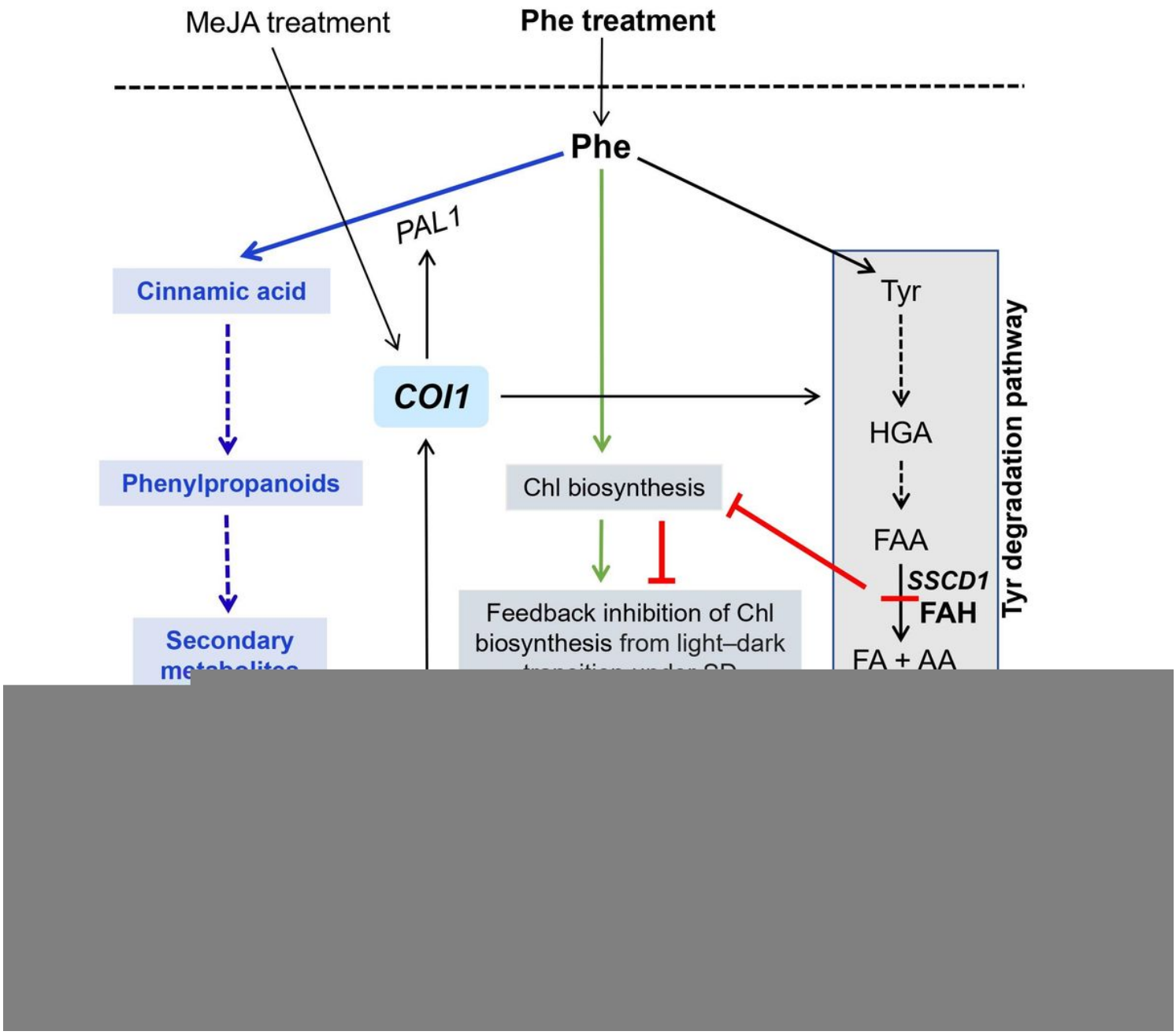

Figure 8

The possible mechanisms by which Phe treatment represses the *sscd1* cell death.

The *SSCD1* gene encodes FAH catalyzing the last step of Tyr degradation pathway. Disruption of FAH in *sscd1* reduces of Chl biosynthesis, which impairs the feedback inhibition of Chl biosynthesis from light–dark transition under SD, leading to the accumulation of ROS and then cell death (red lines and arrows).

Phe treatment promotes Chl biosynthesis, which increases the feedback inhibition of Chl biosynthesis from light–dark transition under SD, reducing ROS and subsequent cell death (green arrows and lines). Meanwhile, Phe treatment activates phenylpropanoid pathway, which reduces ROS and subsequent cell death (blue arrows and lines). In the *sscd1* mutant, ROS induces cell death and the JA production. JA up-regulates Tyr degradation pathway through *COI1*, promoting cell death, however, it also up-regulates *PAL1* through *COI1*, which activates phenylpropanoid pathway, repressing cell death. The effect of MeJA treatment on *sscd1* cell death through phenylpropanoid pathway might be greater than that through Tyr degradation pathway, resulting in that MeJA treatment enhances the repression of *sscd1* cell death by Phe. Arrows indicate induction or positive regulation, whereas lines indicate repression or negative regulation. Arrows with dashed lines indicate multiple steps. FAH, fumarylacetoacetate hydrolase; Chl, chlorophyll; SD, short day; ROS, reactive oxygen species; Phe, phenylalanine; JA, jasmonates; MeJA, methyl jasmonate; HGA, homogentisate; FAA, fumarylacetoacetate; FA, fumarate; AA, acetoacetate.