Deficient plastidic fatty acid synthesis triggers cell death by modulating mitochondrial reactive oxygen species

Jian Wu\(^1,^*\), Yuefeng Sun\(^1,2,^*\), Yannan Zhao\(^1\), Jian Zhang\(^1\), Lilan Luo\(^1\), Meng Li\(^1\), Jinlong Wang\(^1\), Hong Yu\(^1\), Guifu Liu\(^1\), Liusha Yang\(^1\), Guosheng Xiong\(^1\), Jian-Min Zhou\(^1\), Jianru Zuo\(^1\), Yonghong Wang\(^1\), Jiayang Li\(^1\)

\(^1\)State Key Laboratory of Plant Genomics and National Center for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Programmed cell death (PCD) is of fundamental importance to development and defense in animals and plants. In plants, a well-recognized form of PCD is hypersensitive response (HR) triggered by pathogens, which involves the generation of reactive oxygen species (ROS) and other signaling molecules. While the mitochondrion is a master regulator of PCD in animals, the chloroplast is known to regulate PCD in plants. "Arabidopsis" Mosaic Death 1 (MOD1), an enoyl-acyl carrier protein (ACP) reductase essential for fatty acid biosynthesis in chloroplasts, negatively regulates PCD in "Arabidopsis". Here we report that PCD in mod1 results from accumulated ROS and can be suppressed by mutations in mitochondrial complex I components, and that the suppression is confirmed by pharmaceutical inhibition of the complex I-generated ROS. We further show that intact mitochondria are required for full HR and optimum disease resistance to the "Pseudomonas syringae" bacteria. These findings strongly indicate that the ROS generated in the electron transport chain in mitochondria plays a key role in triggering plant PCD and highlight an important role of the communication between chloroplast and mitochondrion in the control of PCD in plants.

Keywords: cell death; PPR protein; ETC; ROS; mitochondria; chloroplasts; "Arabidopsis thaliana"

*These two authors contributed equally to this work.

Introduction

Programmed cell death (PCD) is a genetically regulated process of cell suicide, which is essential for both development and defense response in multicellular organisms [1]. In animals, mitochondria play a central role in initiating PCD by integrating diverse stress signals [2] and the intracellular homeostasis of reactive oxygen species (ROS) is crucial in regulating cell death and cell survival [3]. In plants, several compartments contribute to the formation of ROS, including the plasma membrane, peroxisomes, chloroplasts and mitochondria. ROS synthesized by the plasma membrane NADPH oxidases has been considered to play a role in the hypersensitive response (HR), a type of PCD triggered by pathogens [4]. Chloroplasts are also considered to play an important role in HR since chloroplasts are the major ROS sources under excess of excitation energy conditions [5, 6]. However, a role of mitochondria remains obscure, so does the role of the ROS generated in the mitochondria during cell death in plants. In particular, it remains unclear whether ROS generated in the mitochondria is essential and sufficient to trigger PCD.

In mitochondria, ROS is inevitably produced during ATP synthesis. Several redox centers, mainly complexes I and III, of the mitochondrial electron transport chain (ETC) release electrons to molecular oxygen, serving as the primary source of superoxide formation [7]. Meanwhile, plants have adopted sophisticated mechanisms to remove excessive ROS to avoid the potential damage to cells [8]. Among the ROS scavenging enzymes, superoxide dismutases (SOD) dismutate superoxide anion (O\(_2^−\)) to hydrogen peroxide (H\(_2\)O\(_2\)), which is subsequently converted into H\(_2\)O by catalases or peroxidases [8].

Eukaryotic complex I (NADH:quinone oxidoreduc-
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tase, EC 1.6.5.3) is a mitochondrial redox center assembled by the inner membrane-bound proteins, which catalyzes the transfer of two electrons from NADH to ubiquinone along with the translocation of four protons from the matrix compartment to the intermembrane space. This well-studied large complex contains at least 46 subunits in mammals [9, 10] and more than 49 subunits in plants, 17 of which are unique to plants [11]. Most plant complex I subunits are encoded by the nuclear genome, except for nine (named N4D) which are encoded by the mitochondrial genome [12]. While a few subunits have been partially characterized, the functions of most complex I subunits are still unknown [13]. Of those characterized mutants that carry lesions in subunits of complex I or nuclear-encoded regulators, all show dysfunction of complex I, with various defects in the ETC activity, cellular energy metabolism, ROS homeostasis and stress tolerance [14-23].

The fact that most of the mitochondrial proteins are encoded by the nuclear genome and a small number of proteins by the mitochondrial genome suggests a coordinated regulation of the mitochondrial proteome to maintain their functions. The genome-coordinating mechanisms need reciprocal communications, including anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals [24]. The anterograde control of organelle gene expression is primarily post-transcriptionally regulated by nuclear-encoded regulators. One of these regulators is pentatricopeptide repeat (PPR) proteins, which are characterized by the signature motif of a degenerate 35-amino-acid repeat often arranged in tandem arrays of up to 30 repeats [25, 26]. In animal and fungal genomes, the number of PPR genes is relatively small. However, in plants the size of this gene family is greatly expanded. There are 450 and 477 PPR proteins in Arabidopsis and rice, respectively [27, 28]. Plant PPR proteins are classified into P and PLS subfamilies. The PLS subfamily can be further divided into E, E+ and DYW groups based on their specific C-terminal motifs [27]. Most of the PPR proteins are predicted to target mitochondria or chloroplasts and to bind specific organellar RNAs for posttranscriptional processing, such as RNA editing, splicing, degradation and translation in mitochondria and chloroplasts [24, 26]. The functions of PPR proteins are highly diverse; they may participate in many aspects of plant development, such as embryogenesis and cytoplasmic male sterility.

Our previous studies have shown that the Arabidopsis mosaic death 1 (mod1) is a cell death mutant [29], which results from the deficiency in enoyl-acyl carrier protein (ACP) reductase, a subunit of the fatty acid synthase complex that catalyzes the de novo biosynthesis of fatty acids in plastids. In this paper, we show that the ROS generated in mitochondrial ETC plays a crucial role in triggering the PCD in mod1 originated from the deficiency of fatty acid biosynthesis in plastids.

Results

Accumulation of ROS in mod1

Our previous study showed that the fatty acid biosynthetic mutant mod1 displays pleiotropic phenotypes characteristics of typical PCD features, including irregular cell sizes and shapes, disorganized cellular structures, DNA laddering and consequent cell death [29]. Oxidative burst leading to local ROS accumulation is usually regarded as a critical event associated with plant cell death [30, 31]. We therefore compared the accumulation of ROS (H$_2$O$_2$ and O$_2^-$) between mod1 and wild-type plants by staining with 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively. As shown in Figure 1, both H$_2$O$_2$ and O$_2^-$ are remarkably accumulated in mod1 leaves, suggesting that the accumulation of ROS may play an important role in triggering cell death in mod1.

SOM3 protein is a subunit of the mitochondrial ETC complex I

To elucidate the molecular mechanism underlying cell death in mod1 plants, we screened various suppressors

![Figure 1](image-url)
of mod1 (som) through T-DNA insertion mutagenesis. Among them, a recessive mutant, mod1 som3-1, showed not only a similar morphological phenotype to the wild type (Figure 2A), but also an apparent reduction in cell death (Figure 2B) and in the accumulation of H$_2$O$_2$ (Figure 2C) and O$_2^-$ (Figure 2D). Thus, som3 is indeed a suppressor of mod1.

To clone SOM3, we amplified the inserted T-DNA and its flanking sequences through TAIL-PCR. A T-DNA insertion was found in the first intron of AT1G47260 (Figure 2E), which disturbs the formation of the transcripts of AT1G47260 (Figure 2F), consistent with the recessive inheritance of som3. To verify that suppression of the mod1 phenotypes was a result of the null mutation of AT1G47260, we generated transgenic plants expressing a full-length cDNA of AT1G47260 under the control of the Cauliflower Mosaic Virus 35S promoter (35S). The complemented plants restored the mod1 phenotypes (Figure 2A-2D). Another allele, salk_010194 (som3-2), which contains a T-DNA insertion in the third exon of AT1G47260, also suppressed the phenotypes of mod1 (Figure 2A-2D). Therefore, AT1G47260 is the corresponding SOM3 gene, whose null mutation is responsible for the suppression of the mod1 phenotypes.

To confirm the subcellular localization of SOM3, a 35S:SOM3-GFP transgene was stably expressed in mod1 som3 plants and was capable of restoring the mod1 phenotypes, demonstrating that the SOM3-GFP transgene is fully functional in planta. The GFP fluorescent signal was co-localized with the MitoTracker Red marker (Figure 2G), a dye which specifically stains mitochondria. These data clearly showed that the SOM3 protein is specifically localized in mitochondria, which is consistent with the observations made in previous studies [15, 32, 33]. As expected, MOD1, a key enzyme of fatty acid synthesis, was found as a chloroplast-localized protein (Figure 2H and Supplementary information, Figure S1). The distinctive subcellular localization patterns of SOM3 and MOD1 suggest that the cell death modulated by these two proteins involves an active information exchange between chloroplasts and mitochondria. SOM3 is a subunit of complex I, and disruption of AT1G47260 (SOM3) reduces complex I levels [15]. We also found that the mutation of SOM3 protein, which is undetectable in the som3 mutants (Figure 2I), perturbed the NADH oxidase activities (Figure 2J) of complex I. These results suggested that an intact complex I is possibly required for the MOD1-mediated PCD.

som42 modulates complex I through specifically regulating NAD7

Among other mod1 suppressors, we also identified a
dominant one, mod1 som42, which can partially suppress the mod1 phenotypes (Figure 3A-3D). Molecular characterization of som42 showed that the suppression might result from a T-DNA insertion in the promoter region of AT2g01390 (Figure 3E). Reverse transcription-quantitative-PCR (RT-qPCR) analysis revealed that the expression level of AT2g01390 was significantly increased in mod1 som42 compared with the wild-type plants (Figure 3F), consistent with the gain-of-function mutation nature of som42. Furthermore, overexpression of the AT2g01390 gene showed that the mod1 mutant phenotypes, including plant development, cell death and ROS accumulation, could be completely suppressed (Figure 3A-3D and 3F). Therefore, AT2g01390 is the SOM42 gene, and its overexpression is responsible for the suppression of the mod1 phenotypes.

SOM42 is a nuclear-encoded PPR protein, which belongs to the P subfamily of the PPR family (Supplementary information, Figure S2A) with highly conserved homologous proteins in higher plants (Supplementary in-

Figure 3 Characterization and cloning of som42. (A) Phenotypes of Col-0, mod1, mod1 som42, and mod1 SOM42OE (SOM42 overexpression driven by the 35S promoter in the mod1 background). Scale bar, 2 cm. (B) Leaves stained with Trypan blue. Scale bar, 0.2 cm. (C, D) Seedlings stained with DAB (C) and NBT (D), respectively. Scale bar, 1 cm. (E) Physical map of the T-DNA insertion site. The T-DNA is inserted in the promoter region of AT2G01390, 183 bp upstream of the ATG start codon. (F) The expression levels of SOM42 revealed by RT-qPCR using β-tubulin as reference. Values are means ± SD of three technical replicates, and similar results were obtained in three independent experiments. Statistical differences are indicated with lowercase letters (P < 0.05, one-way ANOVA). (G) Subcellular localization of SOM42-GFP in 35S:SOM42-GFP transgenic plants. MT Red, MitoTracker Red. Scale bar, 20 μm. (H) Northern blot showing the transcription levels of NAD7 probed with exon 3. The blue arrow refers to the mature transcripts of NAD7 and the red arrow refers to the immature transcripts of NAD7. (I) Western blot showing the protein contents of NAD7. (J) Complex I protein stained with Coomassie blue. I, mitochondrial complex I; sub I, mitochondrial sub-complex I. (K) In-gel assay of NADH oxidase activity. I, mitochondrial complex I; sub I, mitochondrial sub-complex I.
Figure 4  Suppression of mod1 cell death by mitochondrial complex I-deficient mutants and rotenone treatment. (A) Phenotypes of mod1, mitochondrial complex I-deficient mutants and their double mutants. Scale bar, 4 cm. (B) Leaves stained with Trypan blue. Scale bar, 0.2 cm. (C) DAB-stained seedlings. Scale bar, 1 cm. (D) NBT-stained seedlings. Scale bar, 1 cm. (E) Phenotypes of Col-0 and mod1 mutant plants following rotenone treatment. Scale bar, 1 cm. (F) Trypan blue-stained leaves of plants following rotenone treatment. Scale bar, 0.2 cm. (G) DAB-stained seedlings following rotenone treatment. Scale bar, 1 cm. (H) NBT-stained seedlings following rotenone treatment. Scale bar, 1 cm.

formation, Figure S2B), some of which have been localized in plastids or mitochondria [18, 27, 34]. As shown in Figure 3G, SOM42, similar to SOM3, was also localized in the mitochondria. In mitochondria, PPR proteins have been found to affect the maturation of complex I NAD transcripts, which are transcribed from the mitochondrial genomes [14, 35, 36]. RT-qPCR analysis showed that NAD7 transcripts were remarkably decreased in both mod1 som42 and SOM42-overexpressing lines (Supplementary information, Figure S3), indicating that SOM42 is specifically involved in the maturation of the NAD7 transcripts, which was further confirmed by northern blot analysis (Figure 3H). Consequently, the accumulation of NAD7 protein was substantially reduced (Figure 3I). It has been reported that the decrease in the NAD7 protein level could impair the abundance and function of com-
plex I [18, 21]. We therefore analyzed the protein level and NADH oxidase activities of complex I, and found that both were apparently reduced in mod1 som42 and severely decreased in SOM42-overexpressing transgenic plants compared with the wild type (Figure 3J and 3K).

Taken together, these results demonstrated that SOM42 negatively regulates complex I activity by modulating the NAD7 transcripts, which encodes a key component of complex I.

Deficiency in complex I suppresses the mod1 phenotypes

To investigate whether the specific reduction of complex I activity is sufficient to suppress the mod1 phenotypes, we first analyzed the effects of the mutations in key components or regulatory factors of complex I, including nMAT1, BIR6 and NDUFS4. The nMAT1 protein is a nuclear maturase that affects NAD1, NAD2 and NAD4 splicing [17, 37], and BIR6 is a PPR protein that affects the splicing of NAD7 intron 1 [18]. NDUFS4 is a nuclear-encoded subunit of complex I [20]. Mutations in these genes cause defects in complex I. We crossed mod1 with nMat1-1 (CS808228), bir6-2 (SALK_000310) and ndufs4-1 (CS825412), respectively, and analyzed the phenotypes of the resulting double mutants. We found that all these double mutations were able to suppress the growth defects (Figure 4A), cell death (Figure 4B) and accumulation of ROS (Figure 4C and 4D) in mod1, which was correlated with the reduction in the NADH oxidase activities of complex I (Supplementary information, Figure S4). It should be pointed out that although the ROS levels of the double mutants between mod1 and individual mitochondrial complex I-deficient mutants including ndufs4-1 were much lower than that of mod1, they were still higher than that of the wild type (Figure

Figure 5 Effects of CSD1 overexpression and knockout of NADPH oxidase genes in the mod1 background. (A) Phenotypes of Col-0, mod1 and 35S:CSD1/mod1 transgenic plants (1-1 and 1-5 independent lines). Scale bar, 1 cm. (B) SOD activities of Col-0, mod1 and 35S:CSD1/mod1 transgenic plants. Statistical differences are indicated with lowercase letters (P < 0.01, one-way ANOVA). Similar results were obtained in three independent experiments. (C) DAB-stained seedlings. Scale bar, 1 cm. (D) NBT-stained seedlings. Scale bar, 1 cm. (E) Leaves stained with Trypan blue. Scale bar, 0.5 cm. (F) Phenotypes of Col-0, mod1, atrbohD atrbohF (atrbohDF) and atrbohDF mod1. Scale bar, 1 cm. (G) Leaves stained with Trypan blue. Scale bar, 0.5 cm. (H) NBT-stained seedlings. Scale bar, 2 cm. See also Supplementary information, Figure S7.
4D and Supplementary information, Figure S5), which is consistent with previous reports [16, 20, 38]. Therefore, it is the mutations in complex I components that cause the suppression of the mod1 phenotypes.

To reinforce the genetic analysis data, we treated mod1 seedlings with rotenone, a specific inhibitor of complex I, which blocks the transfer of electrons from the iron-sulfur centers in complex I to ubiquinone [39]. Treatment with rotenone partially rescued the mod1 phenotypes exemplified by alleviated cell death (Figure 4E, 4F and Supplementary information, Figure S6A) and reduced accumulation of H$_2$O$_2$ and O$_2^-$ in mod1 (Figure 4G, 4H and Supplementary information, Figure S6B), which are similar to those observed in mod1 and complex I double mutants. Taken together, these genetic and physiological studies demonstrated that the mod1-modulated ROS generation and cell death essentially depend on a functional complex I.

**Overexpression of SOD rescues mod1 phenotypes**

Data presented above indicate that mod1-triggered cell death requires a functional complex I, which may be caused by ROS generated from electron transfer. To test this possibility, we attempted to reduce ROS via overexpressing the Arabidopsis cytosolic Cu/Zn SOD (CSD1) [40] in mod1. We found that the mod1 mutant phenotypes were mostly rescued in CSD1-overexpressing transgenic plants (Figure 5A), and the degree of the rescue mod1 phenotypes was well correlated with SOD enzymatic activities (Figure 5B), ROS reduction (Figure 5C and 5D) and attenuation of cell death (Figure 5E). Collectively, these results demonstrated that ROS generated from mitochondrial ETC plays an important role in regulating mod1-triggered cell death.

**ROS generated by NADPH oxidase is not responsible for mod1 cell death**

The Arabidopsis respiratory burst oxidase homolog (AtRBOH) family genes, AtRBOHD and AtRBOHF, have been shown to play a role in the plant defense re-
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We found that the ROS level and mod1 phenotypes including cell death were not apparently affected (Supplementary information, Figure S7). As AtRbohD and AtRbohF are functionally redundant in ROS generation [42], we then generated a triple mutant, atrbohD atrbohF mod1, to compare its phenotypes with those of wild-type, mod1 and mod1 suppressors. As shown in Figure 5F and 5G, the morphology and cell death of the triple mutant plants were highly similar to those of the mod1 mutant plants, although the formation of ROS by NADPH oxidase was blocked in the atrbohD atrbohF double mutant plants (Figure 5H). These results indicate that the ROS generated by the plasma membrane NADPH oxidase is not directly involved in the signaling pathway of mod1 cell death.

Deficiencies in complex I compromise HR and resistance against bacteria

HR is a localized PCD in plants and is tightly associated with ROS. To explore the possible involvement of MOD1-modulated cell death in the defense responses, we examined the HR phenotype of the complex I-deficient mutants challenged by Pseudomonas syringae pv tomato DC3000 (Pst DC3000) carrying avrRpt2 or avrRps4, which are recognized by immune receptors RPS2 or RPS4 [43-45]. We found that all these mutants, including nMat1-1, bir6-2, ndufs4-1 and SOM42-overexpressing transgenic plants, showed attenuated HR induced by both avrRpt2 and avrRps4 (Figure 6A and Supplementary information, Figure S8), strongly suggesting that complex I plays an important role in effector-triggered HR.

We also measured the effect of complex I-deficient mutants on bacterial growth (Figure 6B). Unlike rps2, complex I-deficient mutants were more susceptible to Pst DC3000 harboring an empty vector, indicating that these mutants are compromised in basal resistance to this virulent bacterial strain. The growth of bacteria expressing avrRpt2 or avrRps4 was also enhanced in the complex I-deficient mutants. In addition, ndufs4-1 resembled the fully susceptible rps2 mutant in Pst DC3000(avrRpt2) inoculations and was more susceptible to Pst DC3000 and Pst DC3000(avrRps4) compared to other complex I-deficient mutants. This was consistent with the more severe deficiency of complex I activity in this mutant (Supplementary information, Figure S4). Therefore, deficiency in complex I is most likely involved in basal defense.

Discussion

As a genetically regulated process, PCD is essential for development and defenses in multicellular organisms.
Although mitochondria and mitochondria-derived ROS have been found to play a key role in triggering PCD in animals, their functions are still elusive in plant PCD. In this paper we address these critical questions by isolation and in-depth analyses of som mutants. As summarized in Figure 7, a deficiency in the fatty acid biosynthesis in chloroplasts results in an unidentified signal, which induces the formation of ROS through the mitochondrial ETC to initiate the PCD process in mod1 plants. Mutations or chemicals that impair or block the transfer of electrons along the ETC to form ROS efficiently suppress the phenotypes of mod1 mutant plants, indicating that the ROS generated in mitochondria through ETC plays an essential role in triggering plant PCD.

The identification of both som3 and som42 and the further characterization of their wild-type alleles as a component and a regulator of complex I, respectively, strongly suggest that the mitochondrial complex I plays a critical role in mediating PCD in plants. Mitochondria and chloroplasts are thought to originate from prokaryotes during endosymbiotic evolution in eukaryotic cells. Intimate communications among organelles are necessary to coordinate their activities during growth, development and other physiological processes [24, 46]. In this study, we clearly showed that the chloroplast-controlled cell death is mediated by mitochondria-derived ROS. It is reasonable to assume that an intracellular mobile signal is transported from chloroplasts to mitochondria to trigger PCD, which should be elucidated in the future investigation.

Many nuclear-encoded PPR proteins have been reported to regulate the gene expression of mitochondria-encoded complex I subunits in plants [14, 35, 36, 47]. Here we found that SOM42, a member of P subfamily PPR proteins, specifically affects the mitochondrial complex I subunit NAD7 maturation (Figure 3). The results that in SOM42-overexpressing lines NAD7 transcripts were significantly reduced and other NAD gene transcripts were dramatically increased (Supplementary information, Figure S3) support a feedback control mechanism as previously reported in NAD3 or mETC [22, 23]. Since complex I is the first step in the respiratory redox pathway, it is not surprising that many nuclear-encoded proteins are required to regulate this highly sophisticated complex so that mitochondrial functions can be modulated to accommodate unfavorable environments. The fact that most of the som mutants identified in this study are complex I-deficient mutants (Supplementary information, Figure S9) suggests that mod1 plants provide a suitable genetic tool for screening mutants defective in the subunits of complex I to dissect the function of this complicated complex.

Along the mitochondrial ETC, complex I and complex III are the main source of ROS [7]. The deficiency of complex I will certainly block electron flow into complex III and may reduce ROS generation from complex III, which is supported by treatment of mod1 plants with rotenone, a widely used specific inhibitor of complex I that can induce the formation of ROS from complex I but inhibit the ROS formation from complex III in animals [48, 49]. This result suggests that the ROS generated from the complex III plays a major role in triggering mod1 cell death.

In plants, plasma membrane NADPH oxidases and chloroplasts have been considered to play key roles in the execution of HR [42, 50, 51]. However, direct evidence linking mitochondrial functions to HR or disease resistance is lacking. The finding that the intact mitochondrial ETC is required for the induction of HR upon activation of RPS2 and RPS4, which belong to CC-NB-LRR and TIR-NB-LRR R proteins, respectively [52], suggests that mitochondria play an important role in HR. We also found that the resistance against both virulent and avirulent bacteria is compromised in complex I-deficient mutants. These results indicate that the mitochondria play a role in universal defense signaling. MOD1 is the target of several diphenyl ether toxins, including natural phytotoxins produced by several fungal plant pathogens [53], suggesting that saprotrophic fungi utilize the MOD1-mediated cell death pathway to aid colonization. Taken together, these data suggest that mitochondria play a pivotal role in pathogen resistance. In animals, mitochondria play a fundamental role in triggering different types of cell death involved in immunity and development [54, 55], and dysfunction of the mitochondrial ETC also causes many diseases [56, 57]. Considering that the mechanisms of cell death execution via regulating the mitochondrial complex I are conserved in different organisms, the mitochondrial-dependent cell death pathway in plants may also have important roles in regulating plant development and defense.

Materials and Methods

Plant materials and growth conditions

Seeds of Arabidopsis thaliana wild-type Col-0 and mutants were sterilized and planted on agar plates containing 0.5% MS with 1.0% (w/v) sucrose. Seeds were vernalized for 3-4 days at 4 °C and were placed in growth room. Seedlings (about one week old) were transferred to soil as described previously [29]. The mutants som3-2 (SALK_010194), nMat1-1 (CS808228), birb-2 (SALK_000310), ndufs4-1 (CS825412) and atrbohD/F (CS9558) are T-DNA insertion lines obtained from Arabidopsis Biological Research Center and their genotypes were confirmed by PCR analyses. The primer sequences used for genotyping are listed in
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RNA analysis

Total RNA was prepared from 3-4-week-old seedlings using a TRIZol kit according to the user manual (Cat# 15596-026, Invitrogen) and 12.5 µg of total RNA was treated with DNase I and used for cDNA synthesis with RT kit (Cat# A3500, Promega). RT-qPCR experiments were performed using the Bio-Rad CFX96 System. The primer sequences used for RT-qPCR are listed in Supplementary information, Table S2. RNA gel-blot analysis was carried out as described previously [58]. RNA (20 µg per lane) was separated in a 0.8% (w/v) agarose gel containing 10% (v/v) formaldehyde, blotted onto a Hybond N membrane (Amersham) and probed with the PCR-amplified DNA fragments using specific primers (Supplementary information, Table S2).

Screening for suppressors

The estradiol-inducible activation vector pER16 [59] was introduced into Agrobacterium tumefaciens strain EHA105 by electroporation and Arabidopsis plants were transformed via vacuum infiltration [60]. Transformants (T1 and T2) were selected on plates containing 50 mg/l kanamycin and transferred to soil for pheno-type observation under continuous illumination.

Cloning of gene by TAIL-PCR

The pER16-tagged genomic sequences were recovered by TAIL-PCR [61], and the PCR fragments were analyzed by DNA sequencing. Primers across the T-DNA insert were designed for genotyping, and T-DNA specific primers and arbitrary degenerate primers used for TAIL-PCR are listed in Supplementary information, Table S1.

Trypan blue staining

Trypan blue staining was performed as previously described [62, 63]. Samples were covered with an alcoholic lactophenol trypan blue mixture (30 ml ethanol, 10 g phenol, 10 ml water, 10 ml glyc-erol, 10 ml lactic acid and 10 mg trypan blue), placed in a boiling water bath for 2-3 min, and then left at room temperature for 1 h. The samples were transferred into a chloral hydrate solution (2.5 g/ml) and boiled for 20 min to destain. After multiple exchanges of chloral hydrate solution to reduce the background, samples were equilibrated with 50% (v/v) glycerol, mounted and observed with a stereomicroscope (Olympus SXZ-12).

In situ detection of ROS

In situ detection of O2− and H2O2 were performed as described by Ramel [64] with 4-5-week-old seedlings. For in situ detection of O2−, plantlets were immersed and infiltrated under vacuum with 1 mg/ml NBT (N6876, Sigma-Aldrich) staining solution in potassium phosphate buffer (10 mM) with 10 mM NaN3. After infiltration for 2-3 h, stained plantlets were boiled in acetic acid: glycerol: ethanol (1:1.3, v/v/v) solution for 10 min. Samples were then stored in 95% (v/v) ethanol until scanning. O2− was visualized as a blue color produced by NBT reduction to formazan. For in situ detection of H2O2, the staining agent, DAB (D5637, Sigma-Aldrich), was dissolved in H2O and adjusted to pH 3.8 with HCl. The DAB solution was freshly prepared to prevent auto-oxidation. Samples were immersed and infiltrated under vacuum with 1 mg/ml DAB staining solution. Stained plantlets were then boiled in acetic acid: glycerol: ethanol (1:1.3, v/v/v) solution for 10 min, and then stored in 95% (v/v) ethanol until scanning. H2O2 was visualized as a brown color due to DAB polymerization.

Plasmid construction

To construct plant transformation and transient expression plasmids, fragments containing the full-length CDS of SOM3, SOM42, MOD1, CSD1 and NAD7 were digested and ligated into respective vectors. All the genes, plasmids, primers and the cleavage sites were listed in Supplementary information, Table S3.

Subcellular localization

To obtain transformed Arabidopsis lines, T2 transgenic plants showing a restored phenotype were genetically analyzed and homozygous transgenic lines were selected for in-depth analysis. Staining of mitochondrial-specific dye was performed as described previously [65], and the GFP signal and fluorescence of MitoTracker Red were examined under a confocal microscope at an excitation wavelength of 488 nm and 543 nm, respectively (FluoView 1000, Olympus). To carry out the transient expression analysis of MOD1-GFP, Arabidopsis mesophyll protoplasts were isolated and transfomed as described previously [66], and GFP signals were detected by a confocal fluorescence microscope (FluoView 1000, Olympus).

Assays of enzyme activities of complex I and SOD

Analyses of the NADH oxidase activity and the protein abundance of the mitochondrial complex I were performed as described previously [19]. Proteins of crude membrane extract from 3-4-week-old seedlings were solubilized with 1% (v/v) digitonin and resolved by Blue Native-PAGE. To measure the SOD activity, seedlings were homogenized in liquid nitrogen, then 1 ml extraction buffer consisting of 50 mM sodium phosphate (pH 7.8) and 1% (w/v) polyvinylpyrrolidone was added to 0.1 g material sample. After centrifugation at 10 000 rpm for 5 min at 4 °C, the supernatant containing SOD was used for measuring the SOD activity with the SOD Assay Kit-WST according to the user’s directions (Dojindo).

Inhibitor treatment

The respiration inhibitor rotenone (Sigma-Aldrich) was dis-solved in Dimethyl sulfoxide at concentration of 0.25 M, and then added to MS media at a final concentration of 5, 20 and 40 µM, respectively, and plates or tissue culture bottles were placed in an incubator under continuous illumination at 26 °C.

Preparation of polyclonal antibodies

The CDS of SOM3 or NAD7 was amplified with the primer pair (Supplementary information, Table S3) and subcloned into pET-T28a. Recombinant SOM3 and NAD7 proteins were expressed in BL21 cells and applied to raise polyclonal antibodies in rabbit and mouse, respectively.

Protein extraction and western blot analysis

Arabidopsis seedlings or leaves were grounded into powders in liquid nitrogen and total proteins were extracted using the extraction buffer (500 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 4 M urea, 1 mM phenylmethylsulfonyl fluoride). After the cen trifugation at 13 000 rpm for 10 min at 4 °C, the supernatant was...
transferred into a new tube and quantified by the Bio-Rad protein assay. Protein blots were performed with the SuperSignal West chemiluminescence kit according to the manufacturer’s protocol (Pierce Chemical).

**Bacterial growth and HR assays**

Bacterial growth and HR assays were performed as previously described [67]. Briefly, for bacterial growth assay, 5-week-old *Arabidopsis* leaves under a 10/14 h light/dark photoperiod at 23 °C were infiltrated with *Pst* DC3000 bacteria at 10^5 CFU/ml. The bacterial growth in the leaves was counted at the indicated times. For the HR assay, plants were infiltrated with 10^5 CFU/ml *Pst* DC3000 (avrRpt2) or *Pst* DC3000 (avrRps4).

Additional methods are described in the Supplementary information, Data S1.

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**References**

1. Lam E. Controlled cell death, plant survival and development. *Nat Rev Mol Cell Biol* 2004; 5:305-315.
2. Wang C, Youle RJ. The role of mitochondria in apoptosis. *Annu Rev Genet* 2009; 43:95-118.
3. Marchi S, Giorgi C, Suski JM, et al. Mitochondria-ros crosstalk in the control of cell death and aging. *J Signal Transduct* 2012; 2012:329-635.
4. Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R. Respiratory burst oxidases: the engines of ROS signaling. *Curr Opin Plant Biol* 2011; 14:691-699.
5. Zurbriggen MD, Carrillo N, Hajirezaei MR. ROS signaling in the hypersensitive response: when, where and what for? *Plant Signal Behav* 2010; 5:393-396.
6. Coll NS, Epple P, Dangl JL. Programmed cell death in the plant immune system. *Cell Death Differ* 2011; 18:1247-1256.
7. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. *Biochemistry* 2005; 70:200-214.
8. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 2004; 55:373-399.
9. Fearnley IM, Carroll J, Walker JE. Proteomic analysis of the subunit composition of complex I (NADH:ubiquinone oxidoreductase) from bovine heart mitochondria. *Methods Mol Biol* 2007; 357:103-125.
10. Carroll J, Fearnley IM, Shannon RJ, Hirst J, Walker JE. Analysis of the subunit composition of complex I from bovine heart mitochondria. *Mol Cell Proteomics* 2003; 2:117-126.
11. Klodmann J, Sunderhaus S, Nimtz M, Jansch L, Braun HP. Internal architecture of mitochondrial complex I from *Arabidopsis thaliana*. *Plant Cell* 2010; 22:797-810.
12. Adams KL, Palmer JD. Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. *Mol Phylogen Evol* 2003; 29:380-395.
13. Brandt U. Energy converting NADH:quinone oxidoreductase (complex I). *Annu Rev Biochem* 2006; 75:69-92.
14. Juszczuk IM, Szal B, Rychter AM. Oxidation-reduction and reactive oxygen species homeostasis in mutant plants with respiratory chain complex I dysfunction. *Plant Cell Environ* 2012; 35:296-307.
15. Perales M, Eubel H, Heinemeyer J, Colaneri A, Zabaleta E, Braun HP. Disruption of a nuclear gene encoding a mitochondrial gamma carbonic anhydrase reduces complex I and supercomplex I + III, levels and alters mitochondrial physiology in *Arabidopsis*. *J Mol Biol* 2005; 350:263-277.
16. Lee BH, Lee H, Xiong L, Zhu JK. A mitochondrial complex I defect impairs cold-regulated nuclear gene expression. *Plant Cell* 2002; 14:1235-1251.
17. Nakagawa N, Sakurai N. A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II intron maturase, causes impaired splicing of mitochondrial NAD4 transcript and altered carbon metabolism in *Arabidopsis thaliana*. *Plant Cell Physiol* 2006; 47:772-783.
18. Koprivova A, des Frances-Small CC, Calder G, et al. Identification of a pentatricopeptide repeat protein implicated in splicing of intron 1 of mitochondrial nad7 transcripts. *J Biol Chem* 2010; 285:32192-32199.
19. Pineau B, Layoune O, Danon A, De Paepe R. L-galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I. *J Biol Chem* 2008; 283:32500-32505.
20. Meyer EH, Tomaz T, Carroll AJ, et al. Remodeled respiration in ndufs4 with low phosphorylation efficiency suppresses *Arabidopsis* germination and growth and alters control of metabolism at night. *Plant Physiol* 2009; 151:603-619.
21. Dutilleul C, Garmier M, Noctor G, et al. Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. *Plant Cell* 2003; 15:1212-1226.
22. Zhu Q, Dugardeyn J, Zhang C, et al. The *Arabidopsis thaliana* RNA editing factor SLO2, which affects the mitochondrial electron transport chain, participates in multiple stresses and hormone responses. *Mol Plant* 2014; 7:290-310.
23. Yuan H, Liu D. Functional disruption of the pentatricopeptide protein SLG1 affects mitochondrial RNA editing, plant development, and responses to abiotic stresses in *Arabidopsis*. *Plant J* 2012; 70:432-444.
24. Woodson JD, Chory J. Coordination of gene expression between organellar and nuclear genomes. *Nat Rev Genet* 2008; 9:383-395.
25. Small ID, Peeters N. The PPR motif - a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem Sci* 2000; 25:46-47.
26. Schmitz-Linneweber C, Small I. Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci* 2008; 13:663-670.
27. Lurin C, Andres C, Aubourg S, et al. Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* 2004; 16:2089-2103.
Mitochondria mediate plastid-derived PCD

O'Toole N, Hattori M, Andres C, et al. On the expansion of the pentatricopeptide repeat gene family in plants. Mol Biol Evol 2008; 25:1120-1128.

Mou Z, He Y, Dai Y, Liu X, Li J. Deficiency in fatty acid synthase leads to premature cell death and dramatic alterations in plant morphology. Plant Cell 2000; 12:405-418.

Bestwick CS, Brown IR, Bennett MH, Mansfield JW. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to Pseudomonas syringae pv phaseolicola. Plant Cell 1997; 9:209-221.

Liu Y, Ren D, Pike S, Pallardy S, Gassmann W, Zhang S. Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. Plant J 2007; 51:941-954.

Parisi G, Perales M, Fornasari MS, et al. Gamma carbonic anhydrases in plant mitochondria. Plant Mol Biol 2004; 55:193-207.

Meyer EH, Taylor NL, Millar AH. Resolving and identifying protein components of plant mitochondrial respiratory complexes using three dimensions of gel electrophoresis. J Proteome Res 2008; 7:786-794.

Barkan A, Rojas M, Fuji S, et al. A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. PLoS Genet 2012; 8:e1002910.

Ichinose M, Tasaki E, Sugita C, Sugita M. A PPR-DYW domain within CcmF is required for accumulation of AtrbohD and AtrbohF. Plant and Cell Physiol 2008; 49:391-402.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005; 6:389-402.

Hu Y, Lu W, Chen G, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. Cell Res 2012; 22:399-412.

Sun F, Zhang W, Xiong G, et al. Identification and functional analysis of the MOC1 interacting protein 1. J Genet Genomics 2010; 37:69-77.

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-359.

Bent AF, Kunkel BN, Dahlbeck D, et al. RPS2 of Arabidopsis thaliana: a leucine-rich repeat class of plant disease resistance genes. Science 1994; 265:1856-1860.

Gassmann W, Hinsch ME, Staskawicz BJ. The Arabidopsis RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. Plant J 1999; 20:265-277.

Mindrinos M, Katagiri F, Yu GL, Ausubel FM. The A. thaliana disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell 1994; 78:1089-1099.

Xiao Y, Savchenko T, Baidoo EE, et al. Retrograde signaling by the plastidial metabolite MECPP regulates expression of nuclear stress-response genes. Cell 2012; 149:1525-1535.

Ichinose M, Tasaki E, Sugita C, Sugita M. A PPR-DYW domain within CcmF is required for accumulation of AtrbohD and AtrbohF. Plant and Cell Physiol 2008; 49:391-402.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005; 6:389-402.

Hu Y, Lu W, Chen G, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. Cell Res 2012; 22:399-412.

Sun F, Zhang W, Xiong G, et al. Identification and functional analysis of the MOC1 interacting protein 1. J Genet Genomics 2010; 37:69-77.

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-359.

Dayan FE, Ferreira D, Wang YH, Khan IA, McNROY JA, Pan Z. A pathogenic fungi diphenyl ether phytoxin targets plant enoyl (acyl carrier protein) reductase. Plant Physiol 2008; 147:1062-1071.

Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organisational aging. Science 2011; 333:1109-1112.

Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell 2011; 147:742-758.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005; 6:389-402.

Hu Y, Lu W, Chen G, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. Cell Res 2012; 22:399-412.

Sun F, Zhang W, Xiong G, et al. Identification and functional analysis of the MOC1 interacting protein 1. J Genet Genomics 2010; 37:69-77.

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-359.

Dayan FE, Ferreira D, Wang YH, Khan IA, McNROY JA, Pan Z. A pathogenic fungi diphenyl ether phytoxin targets plant enoyl (acyl carrier protein) reductase. Plant Physiol 2008; 147:1062-1071.

Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organisational aging. Science 2011; 333:1109-1112.

Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell 2011; 147:742-758.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005; 6:389-402.

Hu Y, Lu W, Chen G, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. Cell Res 2012; 22:399-412.

Sun F, Zhang W, Xiong G, et al. Identification and functional analysis of the MOC1 interacting protein 1. J Genet Genomics 2010; 37:69-77.

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-359.

Dayan FE, Ferreira D, Wang YH, Khan IA, McNROY JA, Pan Z. A pathogenic fungi diphenyl ether phytoxin targets plant enoyl (acyl carrier protein) reductase. Plant Physiol 2008; 147:1062-1071.

Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organisational aging. Science 2011; 333:1109-1112.

Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell 2011; 147:742-758.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005; 6:389-402.

Hu Y, Lu W, Chen G, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. Cell Res 2012; 22:399-412.

Sun F, Zhang W, Xiong G, et al. Identification and functional analysis of the MOC1 interacting protein 1. J Genet Genomics 2010; 37:69-77.

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-359.

Dayan FE, Ferreira D, Wang YH, Khan IA, McNROY JA, Pan Z. A pathogenic fungi diphenyl ether phytoxin targets plant enoyl (acyl carrier protein) reductase. Plant Physiol 2008; 147:1062-1071.

Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organisational aging. Science 2011; 333:1109-1112.

Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell 2011; 147:742-758.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005; 6:389-402.

Hu Y, Lu W, Chen G, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. Cell Res 2012; 22:399-412.

Sun F, Zhang W, Xiong G, et al. Identification and functional analysis of the MOC1 interacting protein 1. J Genet Genomics 2010; 37:69-77.

Zuo J, Niu QW, Frugis G, Chua NH. The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-359.

Dayan FE, Ferreira D, Wang YH, Khan IA, McNROY JA, Pan Z. A pathogenic fungi diphenyl ether phytoxin targets plant enoyl (acyl carrier protein) reductase. Plant Physiol 2008; 147:1062-1071.
isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 1995; 8:457-463.

62 Stone JM, Heard JE, Asai T, Ausubel FM. Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) Arabidopsis mutants. Plant Cell 2000; 12:1811-1822.

63 Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 1997; 9:1573-1584.

64 Ramel F, Sulmon C, Gouesbet G, Couée I. Natural variation reveals relationships between pre-stress carbohydrate nutritional status and subsequent responses to xenobiotic and oxidative stress in Arabidopsis thaliana. Ann Bot 2009; 104:1323-1337.

65 Hedtke B, Meixner M, Gillandt S, Richter E, Borner T, Weihe A. Green fluorescent protein as a marker to investigate targeting of organellar RNA polymerases of higher plants in vivo. Plant J 1999; 17:557-561.

66 Yoo SD, Cho YH, Sheen J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2007; 2:1565-1572.

67 Yao J, Withers J, He SY. Pseudomonas syringae infection assays in Arabidopsis. Methods Mol Biol 2013; 1011:63-81.

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