Immune regulators SIB1 and LSD1 antagonistically regulate PhANGs via the GLK transcription factors

Mengping Li^{1,2,a}, Keun Pyo Lee^{1,a}, Tong Liu^{1,2}, Vivek Dogra^{1,3}, Jianli Duan¹,
Mengshuang Li^{1,2}, Weiman Xing¹, and Chanhong Kim^{1,b}

¹ Shanghai Center for Plant Stress Biology and CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, China.

² University of the Chinese Academy of Sciences, Beijing 100049, China.

³ Present address: Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur 176061, India

^a These authors contributed equally to this work.

^b Address correspondence to chanhongkim@sibs.ac.cn

Abstract

GOLDEN2-LIKE (GLK) transcription factors drive the expression of photosynthesis-associated nuclear genes (PhANGs), indispensable for chloroplast biogenesis. We previously demonstrated that the salicylic acid (SA)-induced SIGMA FACTOR-BINDING PROTEIN1 (SIB1), a transcription coregulator and positive regulator of SA-primed cell death, interacts with GLKs. The SIB1-GLK interaction raises the level of light-harvesting antenna proteins in the photosystem II, aggravating photoinhibition and singlet oxygen (¹O₂) burst. ¹O₂ then contributes to SA-primed cell death via EXECUTER1 (EX1, ¹O₂ sensor protein)-mediated retrograde signaling upon reaching a critical level. We now reveal that LESION-SIMULATING DISEASE 1 (LSD1), a transcription coregulator and negative regulator of SA-primed cell death, interacts with GLKs to repress their activities. Consistently, the overexpression of LSD1 represses the expression of PhANGs, but the loss of LSD1 increases their expression. The SA-induced SIB1 then counteractively interacts with GLKs, leading to EX1-mediated cell death. Collectively, we provide a working model that mutually exclusive SA-signaling components SIB1 and LSD1 antagonistically regulate GLKs to fine-tune the expression of PhANGs, priming SA-induced cell death, and sustaining ¹O₂ homeostasis, respectively.

30 Chloroplasts communicate with the nucleus via retrograde signaling (RS) to sustain
 31 photosynthetic homeostasis in response to the ever-changing environment. Upon
 32 exposure to unfavorable environmental conditions, chloroplasts downregulate
 33 photosynthesis-associated nuclear genes (PhANGs), referred to as biogenic RS, but
 34 stimulate the expression of stress-related genes via alternate RS pathways,
 35 collectively called operational RS. The nuclear-encoded chloroplast GENOMES
 36 UNCOUPLED 1 (GUN1) protein plays a pivotal role in the biogenic RS¹. GUN1
 37 integrates various retrograde signals released by the disturbance in plastid gene
 38 expression, reactive oxygen species (ROS) or redox homeostasis, and tetrapyrrole
 39 biosynthesis (TBS) in chloroplasts^{1,2}. The well-known downstream targets of
 40 GUN1-mediated RS are two nuclear genes encoding the GOLDEN2-LIKE (GLK)
 41 transcription factors (TFs)^{3,4}. In fact, GUN1-mediated RS represses *GLK* transcription.
 42 In *Arabidopsis thaliana* (Arabidopsis), GLK1 and GLK2 function redundantly to
 43 express PhANGs, promoting chloroplast biogenesis. Consistently, the loss of GLKs
 44 significantly impairs chloroplast biogenesis⁵.

45 Recent studies discovered an unexpected function of GLKs towards plant
 46 immune responses. The steady-state levels of SA-responsive genes (SARGs) are
 47 significantly lower in GLK1-overexpressing (oxGLK1) Arabidopsis transgenic plants
 48 relative to wild-type (WT) plants⁶. Accordingly, the oxGLK1 plants are susceptible to
 49 the biotrophic pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2, while *glk1*
 50 *glk2* double knockout mutant plants are more resistant compared to WT plants⁷.
 51 However, other studies reported that the GLKs confer resistance towards the cereal
 52 fungal pathogen *Fusarium graminearum*⁶, necrotrophic fungal pathogen *Botrytis*
 53 *cinerea*⁷, and the Cucumber mosaic virus⁸. These findings indicate that there seem to
 54 be multiple regulatory circuits (positive and negative) toward GLK function to
 55 different microbial pathogens. Perhaps, various transcriptional coregulators
 56 distinctively induced (or activated) by pathogens may differently contribute to the
 57 GLK-mediated immune/stress responses.

58 We lately demonstrated that the nuclear-targeted SIGMA FACTOR-BINDING

59 PROTEIN 1 (SIB1), a defense-related transcription coregulator, interacts with GLKs
60 in response to an increase in foliar salicylic acid (SA)⁹. In *Arabidopsis*
61 *lesion-simulating disease 1* (*lsd1*), the increased level of SA rapidly induces the
62 otherwise undetectable SIB1, facilitating its accumulation in both the nucleus and the
63 chloroplasts^{9,10}. The dual targeting of SIB1 was also observed in WT plants after SA
64 treatment. The SIB1-GLK interaction in the nucleus enhanced the expression of
65 PhANGs, while the chloroplast-localized SIB1 (cpSIB1) represses the expression of
66 photosynthesis-associated plastid genes (PhAPGs)⁹. This concurrent uncoupled
67 regulation of PhANGs and PhAPGs increases the levels of singlet oxygen (¹O₂) in
68 chloroplasts, likely through enhanced photoinhibition in PSII⁹. EXECUTER1 (EX1),
69 a ¹O₂ sensor protein¹¹, then mediates ¹O₂-triggered RS to induce cell death in *lsd1*
70 mutant plants⁹. We also demonstrated that the nuclear form of SIB1 (nuSIB1)
71 undergoes co-translational N-terminal acetylation (NTA) and post-translational
72 ubiquitination. While the former case renders nuSIB1 more stable, the latter
73 modification promotes its degradation via the ubiquitin-proteasome system (UPS)
74 within a short period¹². The NTA and UPS seem to finely regulate nuSIB1-mediated
75 stress responses. Nonetheless, earlier reports regarding the positive role of both
76 nuSIB1 and cpSIB1 to SA-primed cell death in *lsd1* mutant suggest that LSD1 may be
77 required to repress the expression of PhANGs to sustain ¹O₂ homeostasis.

78 Here, we demonstrate that LSD1, a transcription coregulator and negative
79 regulator of SA-primed cell death, interacts with GLKs in the nucleus. LSD1
80 considerably reduces the GLK binding activity to the promoters of the examined
81 PhANGs in *Arabidopsis*. In agreement, LSD1-overexpressing plants exhibit
82 significantly reduced levels of PhANGs, while the loss of LSD1 causes a notable
83 upregulation of PhANGs relative to WT plants. Most likely, SA intervenes the
84 LSD1-GLK interaction through a rapid accumulation of nuSIB1, leading to an
85 nuSIB1-GLKs interaction. We thus conclude that the defense-associated but mutually
86 exclusive transcription coregulators nuSIB1 (positive regulator) and LSD1 (negative
87 regulator) antagonistically regulate the expression of PhANGs through GLKs. Such a
88 regulatory circuit may play an essential role in sustaining ¹O₂ and photosynthetic

homeostasis under certain stress conditions wherein SA signaling primes plant stress responses.

Results

LSD1 interacts with the GOLDEN2-LIKE transcription factors GLK1 and GLK2

The stress hormone SA primes cell death in the *lsd1* mutant in a light-dependent manner, a typical characteristic of most lesion mimic mutants (LMMs)^{9,13}. Upon exposure to various stimuli, including light, cold, UV-C, red light, hypoxia, and pathogens¹³⁻²⁰, *lsd1* mutant plants exhibit an uncontrolled foliar cell death, called runaway cell death (RCD). The SA-induced transcription coregulator nuSIB1 potentiates the expression of PhANGs and immune-related genes by modulating the TF activity of GLKs and WRKY33, respectively. Both GLK-nuSIB1 and WRKY33-nuSIB1 interactions contribute to *lsd1* RCD^{9,10}. Alternatively, this finding suggests a possible antagonism between LSD1 and nuSIB1 because nuSIB1-driven immune responses occur in the absence of LSD1.

To reveal the LSD1 interactome, a potential target of nuSIB1, we generated Arabidopsis WT transgenic plants overexpressing GREEN FLUORESCENT PROTEIN (GFP)-tagged LSD1 under the control of the CaMV 35S promoter (35S). The immunoblot assay detected the LSD1-GFP fusion protein at the predicted molecular mass of approximately 46 kD using an anti-GFP antibody (Supplementary Fig. 1). Next, using magnetic beads conjugated with GFP antibody, we co-immunoprecipitated LSD1-GFP and its putative associated proteins. The trypsin-digested protein samples were then subjected to tandem mass spectrometry (MS) analyses. The two independent experiments of co-immunoprecipitation (Co-IP) coupled to MS analysis using two separately prepared biological samples identified 55 putative LSD1-associated proteins, which are absent in the protein samples of GFP-overexpressing transgenic plants (35S:GFP) (Supplementary Table 1).

Among the 55 proteins, we identified GLK1. In Arabidopsis, GLK1 and its

homolog GLK2 share around 50% amino acid sequence identity. Both contain two conserved domains, the DNA-binding domain (DBD) and the C-terminal GCT-box that is explicit in GLK TFs (Fig. 3a)^{5,21}. A comparison of the DBD domain and the GCT-box between GLK1 and GLK2 shows a 90% and 79% identity, respectively²². Therefore, not only GLK1 but also GLK2 may interact with LSD1. To this end, we performed a bimolecular fluorescence complementation (BiFC) assay in *Nicotiana benthamiana* (*N. benthamiana*) leaves. Consistent with the previous report¹⁵, we confirmed the LSD1-LSD1 interaction in the nucleus, as evident in the overlapped signals detected from the YELLOW FLUORESCENT PROTEIN (YFP) and the blue-fluorescent DNA stain 4', 6-diamidino-2-phenylindole (DAPI) (Fig. 1a). Similarly, we observed an equivalent yellow fluorescence signal of YFP in *N. benthamiana* leaf epidermal cells coexpressing LSD1-YFP^N and GLK1 (or GLK2)-YFP^C (Fig. 1a). All YFP signals were exclusively observed in the nucleus (Fig. 1a). The Co-IP and an ensuing immunoblot assay further corroborated the LSD1-GLK interaction (Fig. 1b). We also purified the full-length recombinant proteins of LSD1, GLK1, and GLK2 expressed in *Escherichia coli*. The following gel filtration assays demonstrated that either GLK1 (Fig. 2a) or GLK2 (Fig. 2b) form a complex with LSD1, as shown by their co-migration.

GLK interacts with LSD1 via the conserved region of the C-terminus

In GLKs (Fig. 3a), the C-terminal GCT-boxes drive the homo- or hetero-dimerization in maize²¹. In Arabidopsis, the *turnip yellow mosaic virus* (TYMV) protein P69 interacts with GLK1 and GLK2 proteins through the GCT-box to hinder their activities, leading to the repression of PhANGs and an impaired chloroplast biogenesis²³. To define the necessary GLK region for its interaction with LSD1, we attempted to produce truncated GLK1 and GLK2 recombinant proteins. However, unlike GLK2 protein, we could not produce sufficient quantities of the truncated GLK1 variants retaining only the C-terminal conserved regions. Hence, as indicated in Fig. 3a, we used the recombinant proteins of the truncated GLK2 variants for the *in vitro* gel filtration assays. The results showed that the C-terminus containing the small

conserved region (Fig. 3a) is sufficient to interact with LSD1 (Fig. 3b). To validate this result *in vivo*, we generated various BiFC constructs using both GLK1 and GLK2. GFP-fused GLK1 and GLK2 proteins, intact versus the C-terminus-deleted (including the small conserved region) GLKs (GLK1_{ΔCT} and GLK2_{ΔCT}), were transiently expressed in *N. benthamiana* leaves for their subcellular localization. The confocal images clearly showed no impact of the C-terminus deletion towards a nuclear-localization (Fig. 4a). The subsequent BiFC (Fig. 4b) and Co-IP (Fig. 4c) analyses verified the *in vitro* results indicating that the C-terminal part, including the small conserved region, is essential for the interaction with LSD1.

Loss of LSD1 upregulates GLK target genes

What could be the biological consequence of the LSD1-GLK interaction? Given that GLKs promote the expression of PhANGs³, it is plausible that the loss of LSD1 may primarily affect the expression of GLK target genes. Presumably, it may also participate in the *lsd1* RCD. To find the affected genes either by the loss of LSD1 or of GLK1/GLK2, we compared the RNA sequencing (RNA-seq) data of *lsd1* (*lsd1* versus WT)⁹ and *glk1 glk2* (*glk1 glk2* versus WT)²³. As shown in Fig. 5a, one group of 91 genes (listed in Supplementary Data 1) are shared between the upregulated genes (395, at least twofold) in *lsd1* (Supplementary Data 2) and the downregulated genes (936, at least twofold) in *glk1 glk2* (Supplementary Data 3). When analyzing these 91 genes, looking at the biological processes, photosynthesis and light-harvesting in PSII are over-represented (*P*-value=1.42E-07) (Fig. 5b, Supplementary Data 4). The potentiated expression of PhANGs in *lsd1* versus WT plants is indicative of a negative role of LSD1 in GLK activity. To this end, we also examined the relative transcript abundance of PhANGs in LSD1-overexpressing Arabidopsis transgenic plants (hereafter *oxLSD1*) using reverse transcription quantitative PCR (RT-qPCR). The results indicated that the examined genes encoding light-harvesting chlorophyll *a/b* binding proteins (LHCs in PSII and LHCA1 in PSI) were substantially repressed in *oxLSD1* relative to WT plants (Fig. 5c). *oxLSD1* plants exhibited comparable levels of *GLK1* and *GLK2* transcripts relative to WT (Supplementary Fig. 2), implying that the

175 reduced expression of PhANGs likely results from a post-translational regulation of
176 GLKs.

177 **LSD1 inhibits the DNA-binding activity of GLKs**

178 Given that nuclear-localized LSD1 acts as a transcription coregulator and that LSD1
179 interacts with GLKs, it is conceivable that LSD1 might directly regulate the GLK
180 DNA-binding activity. To explore this assumption, we performed a chromatin
181 immunoprecipitation (ChIP) coupled with a qPCR analysis. The relative activity of
182 GLK1 towards its target promoters was examined in the presence or absence of LSD1
183 using protoplasts isolated from the rosette leaves of *lsd1 glk1 glk2* triple mutant plants.
184 It should be noted that both LSD1 and GLK1 are unstable, and even in 35S
185 promoter-driven *GLK1* stable transgenic lines it is challenging to detect the
186 protein^{24,25}. Thus, we used the protoplast transient expression system to ensure a
187 sufficient protein expression of both LSD1 and GLK1 to elucidate the impact of
188 LSD1 on GLK1 function. The *35S:GLK1-4xMyc* was transiently coexpressed with
189 either *35S:RED FLUORESCENT PROTEIN (RFP)* or *35S:LSD1-RFP* in the
190 protoplasts. ChIP assays were then conveyed with the nuclear lysis from the
191 transfected protoplasts. The agarose beads conjugated with anti-Myc antibody were
192 used to pull down the Myc-tagged protein-DNA complex. The immunoprecipitated
193 DNA was then analyzed using qPCR to compare the DNA-binding activity of GLK1
194 in the presence or absence of LSD1. Finally, we examined the relative expression of
195 well-established GLK target genes such as *LHCB1.4*, *LHCB3*, and *LHCB6*³. The
196 results demonstrated that the presence of LSD1 markedly reduced the DNA-binding
197 activity of GLK1 to the promoters of the *LHCBs* genes (Fig. 5d).

198 **LSD1-GLK interaction regulates chlorophyll biosynthesis in WT**

199 We next validated the above ChIP assay result *in planta*. Considering the positive
200 regulation of chlorophyll synthesis by GLK1³ and the repression of GLK activity by
201 LSD1 (Fig. 5a-d), it is tempting to hypothesize that loss of LSD1 might increases the
202 rate of chlorophyll biosynthesis. By revisiting the previously published RNA-seq

data⁹, we noticed that a set of chlorophyll synthesis genes including *glutamyl tRNA*
reductase (HEMA1/Glu-TR), *genome uncoupled 4 (GUN4)*, *magnesium chelatase H*
subunit (GUN5/CHLH), *magnesium protoporphyrin IX monomethyl ester cyclase*
(CHL27/CRD1), *protochlorophyllide oxidoreductase A (PORA)*, and *chlorophyllide a*
oxygenase (CAO) were markedly upregulated in *lsd1* before the onset of RCD (Fig.
6a,b). We then analyzed the aminolevulinic acid (δ -ALA, the common precursor of all
tetrapyrroles) synthesis rate in light-grown *lsd1* mutant plants treated with levulinic
acid (LA)²⁶, a competitive chemical inhibitor of δ -ALA dehydratase that catalyzes the
synthesis of porphobilinogen through the asymmetric condensation of two δ -ALA
molecules. It is important to note that the δ -ALA synthesis rate is also referred to as
the relative chlorophyll synthesis rate^{27,28} (Fig. 6a). In view of that FLUORESCENT
(FLU) protein directly represses Glu-TR activity to inhibit protochlorophyllide
(Pchlde) accumulation in the dark²⁹ (Fig. 6a) and that *flu* mutant plants exhibit a
higher δ -ALA synthesis rate in the presence of LA²⁹, we used the *flu* mutant as a
positive control.

Interestingly, the δ -ALA levels were almost comparable in *lsd1* and *flu* seedlings.
The concurrent inactivation of both *FLU* and *LSD1* further increased the δ -ALA level
(Fig. 6c). Since the Pchlde levels in the dark would indirectly reflect the rate of
chlorophyll biosynthesis due to the absence of the enzyme(s) that catalyzes Pchlde
turnover³⁰, we measured the Pchlde levels in dark-incubated plants of WT, *flu*, *lsd1*,
and *lsd1 flu* using high-performance liquid chromatography analysis. Pchlde was
highly upregulated in the plants lacking FLU in the dark, as demonstrate earlier³¹ (Fig.
6d). It was remarkable that the loss of LSD1 raises the level of Pchlde
(approximately 1.5-fold increase) in both WT and *flu* mutant backgrounds,
corresponding with the positive effect of the *lsd1* mutation on the δ -ALA synthesis.
However, the total chlorophyll levels between these genotypes were almost
comparable (Supplementary Fig. 3). Considering that chlorophyll synthesis and
catabolism pathways synchronously coordinate chlorophyll homeostasis³², an excess
amount of chlorophyll may immediately undergo degradation. Collectively, these

232 results corroborate the negative role of LSD1 towards GLK activity, which seems to
233 be, at least in part, required for tetrapyrrole homeostasis.

234 **SA-induced SIB1 intervenes LSD1-GLK1 interaction**

235 Since SIB1-mediated genomes uncoupled expression of PhANGs and PhAPGs
236 largely contributes to the *lsd1* RCD⁹, we hypothesized that nuSIB1 would
237 counteractively modulate the LSD1-GLK interaction to reinforce the expression of
238 PhANGs. Considering its prompt turnover via UPS-mediated proteolysis¹², nuSIB1
239 may rapidly intervene the LSD1-GLK interaction, resulting in nuSIB1-GLK
240 interaction and reinforced expression of PhANGs, thereby contributing to cell death⁹.
241 Alternatively, SA per se may interfere with the LSD1-GLK interaction, for instance,
242 through the alteration of the protein conformation of LSD1 or GLKs or both. In fact, a
243 previous report showed a redox-sensitive reconfiguration of LSD1 and a concurrent
244 change of its interactome^{15,33}. Thus, we examined the effect of SA on the
245 LSD1-GLK1 interaction in Arabidopsis leaf protoplasts isolated from WT and the
246 *sib1* mutant. We noticed that SA significantly hindered the LSD1-GLK1 interaction in
247 WT but not in the *sib1* mutant (Fig. 7a), indicating that nuSIB1 rather than SA per se
248 intervenes the LSD1-GLK1 interaction. To further elucidate the antagonistic action of
249 nuSIB1 towards LSD1-GLK1 interaction, the dose-dependent impact of nuSIB1 was
250 examined. For this, LSD1-GFP and GLK1-Myc were transiently coexpressed in
251 Arabidopsis leaf protoplasts, along with different amounts of free RFP or SIB1-RFP.
252 It should be noted that increasing doses of RFP or SIB1-RFP decline the expression of
253 LSD1-GFP and GLK1-Myc, probably due to the diminished transfection efficiency by
254 the excess amounts of constructs (Fig. 7b). Nonetheless, the relative amount of
255 GLK1-Myc protein co-immunoprecipitated with LSD1-GFP was quantified using
256 ImageJ following immunoblot analysis (Fig. 7c). The results showed a SIB1
257 dose-dependent inhibition of the LSD1-GLK1 interaction.

258 It is possible that nuSIB1 also interacts with LSD1 and the then released GLKs
259 may interact with nuSIB1, promoting the expression of PhANGs. However, while the
260 interaction between LSD1-GFP and LSD1-RFP was apparent, no interaction between

LSD1-RFP and SIB1-RFP was observed (Supplementary Fig. 4). It is likely that SA-induced nuSIB1 counteractively interacts with GLKs, which consequently enhances the level of $^1\text{O}_2$ and thereby activates an EX1-mediated cell death response (Fig. 7d). The rapid turnover of nuSIB1 via UPS¹² may turn LSD1-GLK interaction, repressing the expression of PhANGs and GLK-mediated immune responses⁶⁻⁸. Such a dynamic regulatory circuit may be critical in sustaining photosynthetic and ROS homeostasis as well as SA-induced stress responses.

Discussion

LSD1 is present in both cytosol and nucleus. Cytosolic LSD1 is considered functioning as a scaffold protein holding its target proteins under non-stress conditions. According to earlier reports, LSD1-retained proteins include BASIC LEUCINE ZIPPER10 (bZIP10) and METACASPASE1 (MC1), which participate in inducing cell death in the Arabidopsis *lsd1* mutant and during plant-pathogen interaction^{34,35}. Increased cellular stress levels seem to release the LSD1-retained proteins, facilitating cell death response. LSD1 also interacts with the plasma membrane protein GSH-induced LITAF domain protein (GILP), which serves as a negative regulator of cell death³⁶. Neither a recent study³³ which intended to identify LSD1-associated proteins using the tandem affinity purification (TAP) assay nor our IP-MS assay identified the positive or negative regulators of cell death. The TAP assay and IP-MS analysis found only one common protein, UDP-glucose 6-dehydrogenase family protein³³ (Supplementary Table 1). We also identified catalase 3 (CAT3), a detoxifier of ROS, which interaction with LSD1 was identified via IP-MS analysis by another group³⁷ (Supplementary Table 1). CAT3 appears to antagonize *lsd1* RCD³⁷. At present, we reasoned that such inconsistent identification of LSD1-associated proteins by several groups is likely related to the different source materials (e.g. cell culture versus plants), methods, and transient or weak interaction with LSD1. Nonetheless, previous studies on LSD1 and our findings support the increasing notion that LSD1 functions 1. as a scaffold protein in the cytosol to inhibit positive regulators of cell death (bZIP10 and MC1), 2. as a stimulator of the negative regulators of cell death (GILP and CAT3), and 3. as a transcription coregulator in the nucleus.

292 SIB1, a transcription coregulator, rapidly accumulates in both the nucleus and
 293 chloroplasts in response to an increase in foliar SA⁹. The nuSIB1 interacts with GLKs
 294 to stimulate their activity, resulting in the elevated expression of PhANGs, while the
 295 cpSIB1 downregulates the expression of PhAPGs. This SIB1-driven molecular
 296 episode, a so-called “genomes-uncoupled expression”, directs a ¹O₂ burst in the
 297 chloroplasts because of photoinhibition (resulted from the excess light energy and
 298 reduced photosynthetic capacity) and contributes to the *lsd1* RCD through the
 299 activation of EX1-mediated RS⁹. We now found that LSD1 also functions as a
 300 negative regulator of GLKs. The LSD1-GLK interaction in the nucleus (Figs 1 and 2)
 301 led to the repression of PhANGs, especially genes encoding LHCBs and chlorophyll
 302 biosynthesis enzymes (Fig. 5). By contrast, loss of LSD1 resulted in a higher
 303 expression of PhANGs and an increase in TBS in the Mg-branch as compared to WT
 304 plants (Fig. 6). The impact of loss- and gain-of-function of LSD1 towards the
 305 transcript abundances of GLK target genes suggest a steady-state LSD1-GLK
 306 interaction in WT plants grown under normal growth conditions (Figs. 5a-c and 6a,b).
 307 Given that the elevated expression of PhANGs directed by SIB1-GLK interaction
 308 stimulates *lsd1* RCD⁹, it is rational to assume that the SA-induced nuSIB1 interferes
 309 with the LSD1-GLK interaction to ensure a SIB1-GLK-mediated expression of
 310 PhANGs. Indeed, the Co-IP/immunoblot assay confirmed the negative impact of
 311 nuSIB1 on the LSD1-GLK interaction (Fig. 7a-c). We inferred that such opposing
 312 regulation of GLKs by SIB1 and LSD1 might be vital to maintain ¹O₂ homeostasis in
 313 chloroplasts during and after SA-increasing stress conditions (Fig. 7d).

314 Since both abiotic and biotic stressors promote *lsd1* RCD^{13-17,20,38}, it is
 315 conceivable that the repression of PhANGs by LSD1-GLK interaction would be an
 316 integral part of regulating ¹O₂ and minimizing photodamage in chloroplasts under
 317 various stress conditions. This notion also raises a plausible idea that GUN1-mediated
 318 RS, which transcriptionally represses PhANGs, may further facilitate plant
 319 stress/acclimation responses. Consistently, loss of GUN1 compromises the plant
 320 resistance towards heat, water, drought, cold, and high-light stresses³⁹⁻⁴³. Moreover,

321 *gun1* mutant plants exhibit an increased level of ROS under these stress
322 conditions^{39,41,43}. However, how the GUN1-mediated RS facilitates plant stress
323 tolerance remained an enigma. Now, according to our present studies on SIB1 and
324 LSD1, GLKs may probably play a key role in GUN1-RS-mediated stress responses.
325 Besides, like SIB1, GLK1 also undergoes post-translational regulation through UPS²⁴.
326 It seems that various signaling components, such as GUN1, SIB1, and LSD1, and the
327 posttranslational UPS target GLKs for photosynthesis and ROS homeostasis as well
328 as SA-mediated stress responses.

329 **Methods**

330 **Plant materials and growth conditions**

331 The seeds used in this study were derived from *Arabidopsis thaliana* Columbia-0
332 (Col-0) ecotype and were harvested from plants grown under continuous light (CL;
333 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 22 ± 2 °C. Arabidopsis mutant seeds used in this study, including
334 *lsd1-2* (SALK_042687)⁹ and *glk1 glk2* (*Atglk1.1; Atglk2.1*)⁵ were obtained from the
335 Nottingham Arabidopsis Stock Centre (NASC). *flu5c* has been described previously³¹.
336 The double and triple mutants in the *lsd1-2* background including *lsd1 flu* and *lsd1*
337 *glk1 glk2* were generated by crossing the homozygous plants. The genotypes of all
338 mutants were confirmed by PCR-based analyses. Primer sequences used for PCR are
339 listed in Supplementary Table 2.

340 Seeds were surface sterilized with 70% (v/v) ethanol containing 0.05% (v/v)
341 Triton X-100 (Sigma-Aldrich) for 5 min and washed five times with sterile distilled
342 water. The sterile seeds were plated on Murashige and Skoog (MS) medium (Duchefa
343 Biochemie) with 0.7% (w/v) agar (Duchefa Biochemie) and stratified at 4 °C in
344 darkness for two days prior to placing in a growth chamber (CU-41L4; Percival
345 Scientific) with CL condition.

346 **Generation of *LSD1* overexpression line**

347 The stop-codon-less *LSD1* coding sequence (CDS) was cloned into the modified

348 pCAMBIA3300 binary vector containing the 35S promoter, a NcoI restriction site,
349 and the *EGFP*. Arabidopsis stable transgenic lines were generated by a floral dip
350 transformation procedure⁴⁵ with *Agrobacterium tumefaciens* strain GV3101.
351 Homozygous transgenic lines were selected on MS medium containing 12.5 mg/l
352 glufosinate-ammonium (Sigma-Aldrich).

353 **RNA extraction and RT-quantitative PCR (RT-qPCR)**

354 Total RNA was isolated from leaf tissues using the Spectrum Plant Total RNA Kit
355 (Sigma-Aldrich) according to the manufacturer's instructions. The concentration of
356 RNA was determined using the ultraviolet-visible spectrophotometer (NanoDrop™,
357 Thermo Fisher Scientific), and the quality of RNA was evaluated by the measurement
358 of the A260/A280 ratio. cDNA synthesis was performed with 1 µg of total RNA using
359 the PrimeScript™ RT Reagent Kit (Takara) following the manufacturer's instructions.
360 The RT-qPCR was performed on QuantStudio™ Flex Real-Time PCR System
361 (Applied Biosystems) using iTaq Universal SYBR Green PCR master mix (Bio-Rad).
362 The relative transcript level was calculated by the ddCt method⁴⁶ and normalized to
363 the *ACTIN2* (AT3G18780) gene transcript level. The sequences of the primers used
364 for RT-qPCR are listed in Supplementary Table 2.

365 **Co-immunoprecipitation (Co-IP) assay**

366 Co-IP assays were performed using *Nicotiana benthamiana* or Arabidopsis leaf
367 protoplasts transiently coexpressed with the indicated combination of proteins. For the
368 Co-IP assays in *N. benthamiana*, the *35S:LSD1-sGFP*, *35S:GLK1-4xMyc*, and
369 *35S:GLK2-4xMyc* constructs were created as described previously⁹. Briefly,
370 pDONR/Zeo entry vector (Thermo Scientific) containing the stop codon-less
371 full-length CDS of *LSD1*, *GLK1*, or *GLK2* was recombined into the destination vector
372 pGWB605 for C-terminal fusion with sGFP or pGWB617 for C-terminal fusion with
373 4xMyc through the Gateway LR reaction (Thermo Scientific). For the
374 *35S:GLK1-4xMyc* and *35S:GLK2-4xMyc* constructs, a linker DNA encoding
375 Gly-Gly-Ser-Gly-Gly-Ser was added between 4xMyc tag and GLK1 or GLK2 to

increase conformational flexibility of the fusion protein as described previously²⁴. The same procedures were used to create the *35S:GLK1^{ΔCT}-4xMyc* and *35S:GLK2^{ΔCT}-4xMyc* constructs. The different combinations of selected vectors were coexpressed in 4-week-old *Nicotiana benthamiana* leaves by *Agrobacterium*-mediated leaf infiltration as described by *Boruc et al.*⁴⁷. For the Co-IP assays in *Arabidopsis* leaf protoplasts, the *35S:LSD1-sGFP*, *35S:GLK1-4xMyc*, *35S:SIB1-RFP*, and *35S:LSD1-RFP* were cloned into the pSAT6 vector⁴⁸. The isolation and transfection of *Arabidopsis* leaf protoplasts were performed as described previously⁴⁹. The indicated combination of vectors was cotransfected into protoplasts (3×10^6) isolated from 4-week-old plants of WT or *sib1*.

Total protein was extracted using an IP buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5mM EDTA, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40 (NP-40), 1% deoxycholate, 0.1% (w/v) SDS, 1× cOmplete protease inhibitor cocktail (Roche), 1 mM PMSF, and 50 μM MG132. The protein extracts were incubated with 20 μL of GFP-Trap magnetic agarose beads (GFP-TrapMA, Chromotek) for 2 h at 4 °C by vertical rotation (10 rpm). After incubation, the beads were washed five times with the washing buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 50 μM MG132, and 1× cOmplete protease inhibitor cocktail. The immunoprecipitated proteins were then eluted with 2 × SDS protein sample buffer [120 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (v/w) SDS, 0.04% (v/w) bromophenol blue, and 10% (v/v) β-mercaptoethanol] for 10 min at 95 °C. The eluates were subjected to 10% SDS-PAGE gels, and the interaction between coexpressed proteins was examined by immunoblot analyses using a mouse anti-Myc monoclonal antibody (1:10,000; Cell Signaling Technology), a rat anti-RFP monoclonal antibody (1:10,000; Chromotek), and a mouse anti-GFP monoclonal antibody (1:5,000; Roche).

ChIP-qPCR assays

ChIP assays were performed using *Arabidopsis* leaf protoplasts as described

previously^{9,49,50}. Briefly, 1mg of pSAT6 vectors containing *35S:GLK1-4xMyc* DNA were transfected with or without pSAT6 vector containing *35S:LSD1-RFP* DNA into Arabidopsis leaf protoplasts (2×10^7) isolated from 4-week-old *lsd1 glk1 glk2* triple mutant plants grown under 10-h light/14-h dark conditions at a light intensity of 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Afterward, the protoplasts were incubated at 24 °C for 16 h under dim light conditions. The protoplast chromatin was crosslinked by 1% (v/v) formaldehyde in 1 X PBS (pH 7.4) for 10 min and quenched with 0.1 M glycine for 5 min. After isolating nuclei from the protoplasts, the chromatin was sheared by sonication into a major size of around 500 bp. The lysates were diluted with 10× ChIP dilution buffer [1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 50 μM MG132, 1 mM PMSF, and 1× protease inhibitor cocktail] and precleared by incubation with 50 μl Protein-A agarose beads/Salmon sperm DNA (Millipore) at 4 °C for 1 h. The samples were then incubated with anti-Myc monoclonal antibodies (1:10,000; Cell Signaling Technology) at 4 °C overnight. To determine nonspecific binding of DNA on beads, ChIP assays were also performed without antibody. After washing the beads⁵⁰, the immunocomplexes were eluted with elution buffer containing 1% (w/v) SDS and 100 mM NaHCO_3 . The eluates were treated with proteinase K for 1 h at 37 °C after reverse cross-linking. The bound DNA fragments were purified according to *Lee et al.*⁵⁰ and precipitated with ethanol in the presence of glycogen. The purified DNA was dissolved in water. qPCRs were performed on bound and input DNAs. The primers for each tested gene are listed in Supplementary Table 2. The amount of DNA enriched by the anti-Myc antibody was calculated in comparison with the respective input DNA used for each ChIP analysis. Afterward, the enrichment was calculated by normalizing against the corresponding control sample (without antibody).

Production of recombinant proteins

To produce recombinant proteins of LSD1, GLK1, GLK2, and the truncated GLK2 variants (247-386 and 296-386), the coding sequences (CDS) of genes were cloned into the modified pET21b (Novagen) expression vector after adding a cleavage site

for the tobacco etch virus (TEV) protease to the 5' end of the CDSs. The recombinant proteins with a cleavable N-terminal 10xHis-MsyB tag were expressed in *E. coli* BL21 (DE3). After culturing the cells at 37 °C until OD₆₀₀ 0.6, recombinant proteins were induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h at 16 °C. Cells were pelleted by centrifugation and resuspended with buffer A [(50 mM Tris (pH 8.0), 200 mM NaCl, and 1 mM PMSF)]. The cells were lysed by high-pressure homogenizer at 600-800 bar and then centrifuged at 17,000 rpm for 50 min. Each soluble fraction was passed over a Ni-NTA column (Novagen) and eluted with buffer containing 25 mM Tris (pH 8.0), 200 mM NaCl, and 200 mM Imidazole. Subsequently, the eluates containing recombinant proteins with 10xHis-MsyB tag were further purified by anion-exchange column (Source-15Q; GE Healthcare). The 10xHis-MSB tag was cleaved by TEV protease at 4 °C overnight and removed by anion-exchange column. Untagged recombinant proteins were then concentrated and further purified by size-exclusion chromatography (Superdex 200 Increase10/300 GL; GE Healthcare) in buffer containing 20 mM Tris (pH 8.0), 200 mM NaCl and 3 mM DTT. The peak fractions of each protein were pooled together and used for gel filtration assay.

450 **Gel filtration assay**

451 The recombinant proteins purified as described above were subjected to gel filtration
452 assay (Superdex 200 Increase10/300 GL; GE Healthcare) in buffer containing 20 mM
453 Tris (pH 8.0), 200 mM NaCl, and 3 mM DTT. A mixture of the purified LSD1 and
454 GLK1 (or GLK2) proteins was incubated at 4 °C for 1 h before gel filtration assay.
455 Samples from relevant fractions were applied to SDS-PAGE and visualized by
456 Coomassie blue staining. Same procedures were used to examine the interaction of
457 LSD1 with the truncated variants of GLK2 proteins.

458 **Measuring ALA synthesis rate**

459 The ALA synthesis rate was quantified as previously described²⁹. The CL-grown
460 16-day-old plants of WT, *flu*, *lsd1*, and *lsd1 flu* were vacuum-infiltrated for 5 min with

an 80 mM levulinic acid (Sigma) solution containing 10mM KH₂PO₄ (pH 7.2) and 0.5% (v/v) Tween 20. After 1 hour incubation at room temperature under CL, samples were immediately frozen in liquid nitrogen and then homogenized in 4% (v/v) TCA. The homogenates were lysed at 95 °C for 15 min, cooled on ice for 2 min, and filtrated with 0.45 µm cellulose acetate membrane filters (Sterlitech). The filtrated lysates were neutralized with an equal volume of 0.5 M NaH₂PO₄ (pH 7.5). Afterward, ethylacetoacetate (1/5) was added and then the samples incubated at 95 °C for 10 min. After cooling on ice for 5 min, the extracts were mixed with the same volume of fresh Ehrlich's reagent [0.2 g *p*-dimethylaminobenzaldehyde (Sigma-Aldrich), 8.4ml acetic acid, and 1.6 ml 70% (v/v) perchloric acid (Sigma-Aldrich)] and centrifuged at 14,000 g for 5 min (at 4 °C). The OD of each supernatant was measured at 553 nm using the NanoDrop 2000 (Thermo Fisher Scientific). The amount of δ-ALA was calculated using a coefficient of $7.45 \times 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$.

Gene ontology (GO) enrichment analysis

The individual RNA-seq data using the *lsd1* and *glk1 glk2*, analyzed in Fig. 5a, were previously published by *Lv et al.*⁹ and *Ni et al.*²³, respectively. The GO enrichment analysis of the selected genes shown in Supplementary Data 4 was performed on gprofiler at <https://biit.cs.ut.ee/gprofiler> and represents the significantly enriched GO terms in the data set of biological processes (BP) with a significance of *P*-value < 0.05.

Pigment analysis

The levels of chlorophyll and Pchlides were measured in 10-day-old plants of WT, *flu*, *lsd1*, and *lsd1 flu* as described by *Lichtenthaler*⁵¹ and *Goslings et al.*²⁹, respectively.

References

- 1 Nott, A., Jung, H. S., Koussevitzky, S. & Chory, J. Plastid-to-nucleus retrograde signaling. *Annu Rev Plant Biol* **57**, 739-759 (2006).
- 2 Chan, K. X., Phua, S. Y., Crisp, P., McQuinn, R. & Pogson, B. J. Learning the Languages of the Chloroplast: Retrograde Signaling and Beyond. *Annu Rev*

- 489 *Plant Biol* **67**, 25-53 (2016).
- 490 3 Waters, M. T. *et al.* GLK transcription factors coordinate expression of the
- 491 photosynthetic apparatus in Arabidopsis. *Plant Cell* **21**, 1109-1128 (2009).
- 492 4 Martin, G. *et al.* Phytochrome and retrograde signalling pathways converge to
- 493 antagonistically regulate a light-induced transcriptional network. *Nat Commun*
- 494 **7**, 11431 (2016).
- 495 5 Fitter, D. W., Martin, D. J., Copley, M. J., Scotland, R. W. & Langdale, J. A.
- 496 GLK gene pairs regulate chloroplast development in diverse plant species.
- 497 *Plant J* **31**, 713-727 (2002).
- 498 6 Savitch, L. V., Subramaniam, R., Allard, G. C. & Singh, J. The GLK1 'regulon'
- 499 encodes disease defense related proteins and confers resistance to *Fusarium*
- 500 *graminearum* in Arabidopsis. *Biochem Biophys Res Commun* **359**, 234-238
- 501 (2007).
- 502 7 Murmu, J. *et al.* Arabidopsis GOLDEN2-LIKE (GLK) transcription factors
- 503 activate jasmonic acid (JA)-dependent disease susceptibility to the biotrophic
- 504 pathogen *Hyaloperonospora arabidopsidis*, as well as JA-independent plant
- 505 immunity against the necrotrophic pathogen *Botrytis cinerea*. *Mol Plant*
- 506 *Pathol* **15**, 174-184 (2014).
- 507 8 Han, X. Y. *et al.* GOLDEN2-LIKE transcription factors coordinate the
- 508 tolerance to Cucumber mosaic virus in Arabidopsis. *Biochem Biophys Res*
- 509 *Commun* **477**, 626-632 (2016).
- 510 9 Lv, R. *et al.* Uncoupled Expression of Nuclear and Plastid
- 511 Photosynthesis-Associated Genes Contributes to Cell Death in a Lesion Mimic
- 512 Mutant. *Plant Cell* **31**, 210-230 (2019).
- 513 10 Lai, Z. *et al.* Arabidopsis sigma factor binding proteins are activators of the
- 514 WRKY33 transcription factor in plant defense. *Plant Cell* **23**, 3824-3841
- 515 (2011).
- 516 11 Dogra, V., Li, M., Singh, S., Li, M. & Kim, C. Oxidative post-translational
- 517 modification of EXECUTER1 is required for singlet oxygen sensing in
- 518 plastids. *Nat Commun* **10**, 2834 (2019).
- 519 12 Li, Z. *et al.* N-Terminal Acetylation Stabilizes SIGMA FACTOR BINDING
- 520 PROTEIN1 Involved in Salicylic Acid-Primed Cell Death. *Plant Physiol* **183**,
- 521 358-370 (2020).
- 522 13 Muhlenbock, P. *et al.* Chloroplast signaling and LESION SIMULATING
- 523 DISEASE1 regulate crosstalk between light acclimation and immunity in
- 524 Arabidopsis. *Plant Cell* **20**, 2339-2356 (2008).
- 525 14 Jabs, T., Dietrich, R. A. & Dangl, J. L. Initiation of runaway cell death in an
- 526 Arabidopsis mutant by extracellular superoxide. *Science* **273**, 1853-1856
- 527 (1996).
- 528 15 Dietrich, R. A., Richberg, M. H., Schmidt, R., Dean, C. & Dangl, J. L. A novel
- 529 zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as
- 530 a negative regulator of plant cell death. *Cell* **88**, 685-694 (1997).
- 531 16 Muhlenbock, P., Plaszczyca, M., Plaszczyca, M., Mellerowicz, E. & Karpinski,
- 532 S. Lysigenous aerenchyma formation in Arabidopsis is controlled by LESION

- 533 SIMULATING DISEASE1. *Plant Cell* **19**, 3819-3830 (2007).
- 534 17 Huang, X., Li, Y., Zhang, X., Zuo, J. & Yang, S. The Arabidopsis LSD1 gene
535 plays an important role in the regulation of low temperature-dependent cell
536 death. *New Phytol* **187**, 301-312 (2010).
- 537 18 Karpinski, S., Szechynska-Hebda, M., Wituszynska, W. & Burdiak, P. Light
538 acclimation, retrograde signalling, cell death and immune defences in plants.
539 *Plant Cell Environ* **36**, 736-744 (2013).
- 540 19 Chai, T., Zhou, J., Liu, J. & Xing, D. LSD1 and HY5 antagonistically regulate
541 red light induced-programmed cell death in Arabidopsis. *Front Plant Sci* **6**,
542 292 (2015).
- 543 20 Rusaczek, A. *et al.* Role of phytochromes A and B in the regulation of cell
544 death and acclimatory responses to UV stress in Arabidopsis thaliana. *J Exp*
545 *Bot* **66**, 6679-6695 (2015).
- 546 21 Rossini, L., Cribb, L., Martin, D. J. & Langdale, J. A. The maize golden2 gene
547 defines a novel class of transcriptional regulators in plants. *Plant Cell* **13**,
548 1231-1244 (2001).
- 549 22 Bravo-Garcia, A., Yasumura, Y. & Langdale, J. A. Specialization of the
550 Golden2-like regulatory pathway during land plant evolution. *New Phytol* **183**,
551 133-141 (2009).
- 552 23 Ni, F. *et al.* Turnip Yellow Mosaic Virus P69 Interacts with and Suppresses
553 GLK Transcription Factors to Cause Pale-Green Symptoms in Arabidopsis.
554 *Mol Plant* **10**, 764-766 (2017).
- 555 24 Tokumaru, M. *et al.* Ubiquitin-Proteasome Dependent Regulation of the
556 GOLDEN2-LIKE 1 Transcription Factor in Response to Plastid Signals. *Plant*
557 *Physiol* **173**, 524-535 (2017).
- 558 25 Waters, M. T., Moylan, E. C. & Langdale, J. A. GLK transcription factors
559 regulate chloroplast development in a cell-autonomous manner. *Plant J* **56**,
560 432-444 (2008).
- 561 26 Nandi, D. L. & Shemin, D. Delta-aminolevulinic acid dehydratase of
562 Rhodospseudomonas spheroides. 3. Mechanism of porphobilinogen synthesis. *J*
563 *Biol Chem* **243**, 1236-1242 (1968).
- 564 27 Beale, S. I. & Castelfranco, P. A. The Biosynthesis of delta-Aminolevulinic
565 Acid in Higher Plants: I. Accumulation of delta-Aminolevulinic Acid in
566 Greening Plant Tissues. *Plant Physiol* **53**, 291-296 (1974).
- 567 28 Hou, Z., Yang, Y., Hedtke, B. & Grimm, B. Fluorescence in blue light (FLU)
568 is involved in inactivation and localization of glutamyl-tRNA reductase during
569 light exposure. *Plant J* **97**, 517-529 (2019).
- 570 29 Goslings, D. *et al.* Concurrent interactions of heme and FLU with Glu tRNA
571 reductase (HEMA1), the target of metabolic feedback inhibition of
572 tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants. *Plant J*
573 **40**, 957-967 (2004).
- 574 30 Forreiter, C. & Apel, K. Light-independent and light-dependent
575 protochlorophyllide-reducing activities and two distinct
576 NADPH-protochlorophyllide oxidoreductase polypeptides in mountain pine

- 577 (Pinus mugo). *Planta* **190**, 536-545 (1993).
- 578 31 Meskauskiene, R. *et al.* FLU: a negative regulator of chlorophyll biosynthesis
- 579 in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **98**, 12826-12831 (2001).
- 580 32 Tanaka, R., Kobayashi, K. & Masuda, T. Tetrapyrrole Metabolism in
- 581 *Arabidopsis thaliana*. *Arabidopsis Book* **9**, e0145 (2011).
- 582 33 Czarnocka, W. *et al.* The dual role of LESION SIMULATING DISEASE 1 as
- 583 a condition-dependent scaffold protein and transcription regulator. *Plant Cell*
- 584 *Environ* **40**, 2644-2662 (2017).
- 585 34 Kaminaka, H. *et al.* bZIP10-LSD1 antagonism modulates basal defense and
- 586 cell death in *Arabidopsis* following infection. *EMBO J* **25**, 4400-4411 (2006).
- 587 35 Coll, N. S. *et al.* *Arabidopsis* type I metacaspases control cell death. *Science*
- 588 **330**, 1393-1397 (2010).
- 589 36 He, S. *et al.* The LSD1-interacting protein GILP is a LITAF domain protein
- 590 that negatively regulates hypersensitive cell death in *Arabidopsis*. *PLoS One* **6**,
- 591 e18750 (2011).
- 592 37 Li, Y., Chen, L., Mu, J. & Zuo, J. LESION SIMULATING DISEASE1
- 593 interacts with catalases to regulate hypersensitive cell death in *Arabidopsis*.
- 594 *Plant Physiol* **163**, 1059-1070 (2013).
- 595 38 Torres, M. A., Jones, J. D. & Dangl, J. L. Pathogen-induced, NADPH
- 596 oxidase-derived reactive oxygen intermediates suppress spread of cell death in
- 597 *Arabidopsis thaliana*. *Nat Genet* **37**, 1130-1134 (2005).
- 598 39 Cheng, J. *et al.* Plastid signals confer *Arabidopsis* tolerance to water stress. *Z*
- 599 *Naturforsch C* **66**, 47-54 (2011).
- 600 40 Miller, G. *et al.* Double mutants deficient in cytosolic and thylakoid ascorbate
- 601 peroxidase reveal a complex mode of interaction between reactive oxygen
- 602 species, plant development, and response to abiotic stresses. *Plant Physiol* **144**,
- 603 1777-1785 (2007).
- 604 41 Tang, H. *et al.* Plastid signals induce ALTERNATIVE OXIDASE expression
- 605 to enhance the cold stress tolerance in *Arabidopsis thaliana*. *Plant Growth*
- 606 *Regul* **74**, 275-283 (2014).
- 607 42 Zhang, Z. W. *et al.* The roles of two transcription factors, ABI4 and CBFA, in
- 608 ABA and plastid signalling and stress responses. *Plant Mol Biol* **83**, 445-458
- 609 (2013).
- 610 43 Zhang, Z. W. *et al.* Mg-protoporphyrin, haem and sugar signals double cellular
- 611 total RNA against herbicide and high-light-derived oxidative stress. *Plant Cell*
- 612 *Environ* **34**, 1031-1042 (2011).
- 613 44 Lee, K. P., Kim, C., Landgraf, F. & Apel, K. EXECUTER1- and
- 614 EXECUTER2-dependent transfer of stress-related signals from the plastid to
- 615 the nucleus of *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **104**,
- 616 10270-10275 (2007).
- 617 45 Clough, S. J. & Bent, A. F. Floral dip: a simplified method for
- 618 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**,
- 619 735-743 (1998).
- 620 46 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data

- 621 using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.
- 622 *Methods* **25**, 402-408 (2001).
- 623 47 Boruc, J. *et al.* Functional modules in the Arabidopsis core cell cycle binary
- 624 protein-protein interaction network. *Plant Cell* **22**, 1264-1280 (2010).
- 625 48 Tzfira, T. *et al.* pSAT vectors: a modular series of plasmids for autofluorescent
- 626 protein tagging and expression of multiple genes in plants. *Plant Mol Biol* **57**,
- 627 503-516 (2005).
- 628 49 Yoo, S. D., Cho, Y. H. & Sheen, J. Arabidopsis mesophyll protoplasts: a
- 629 versatile cell system for transient gene expression analysis. *Nat Protoc* **2**,
- 630 1565-1572 (2007).
- 631 50 Lee, J. H., Jin, S., Kim, S. Y., Kim, W. & Ahn, J. H. A fast, efficient chromatin
- 632 immunoprecipitation method for studying protein-DNA binding in
- 633 Arabidopsis mesophyll protoplasts. *Plant Methods* **13**, 42 (2017).
- 634 51 Lichtenthaler, H. K. Chlorophylls and Carotenoids - Pigments of
- 635 Photosynthetic Biomembranes. *Method Enzymol* **148**, 350-382 (1987).
- 636

637 **Acknowledgements**

638 We thank the Core Facility of Proteomics in Shanghai Center for Plant Stress Biology
639 (PSC) for carrying out mass spectrometry. This research was supported by the
640 Strategic Priority Research Program from the Chinese Academy of Sciences (Grant
641 No. XDB27040102), the 100-Talent Program of the Chinese Academy of Sciences,
642 and the National Natural Science Foundation of China (NSFC) (Grant No. 31871397)
643 to C.K..

644 **Author contributions**

645 M.L., K.P.L., T.L., V.D., W.X., and C.K. designed the experiments. M.L., K.P.L., T.L.,
646 V.D., J.D., and M.S.L. performed the experiments. M.L. K.P.L., T.L., V.D., W.X., and
647 C.K. analyzed the data. M.L., K.P.L., and C.K. wrote the manuscript. All authors
648 discussed the results and reviewed the manuscript.

649 **Competing interests:** The authors declare no competing interests.

650 **Supplementary Information:**

651 Supplementary Figures 1-4

652 Supplementary Tables 1 and 2

653 Supplementary Data 1-4

654 **Figure legends**

655 **Figure 1. LSD1 interacts with GLK1 and GLK2 *in vivo*.** **a**, Bimolecular
656 fluorescence complementation (BiFC) assays. The GLK1 and GLK2 fused with
657 C-terminal part of YFP (YFP^C) were individually coexpressed with the N-terminal
658 part of the YFP (YFP^N) tagged with LSD1 in *N. benthamiana* leaves. The
659 combinations of LSD1-YFP^N+LSD1-YFP^C and LSD1-YFP^N+YFP^C were used as a
660 positive and negative control, respectively. DAPI was used to stain the nucleus. All
661 images were taken under the same scale (scale bars: 25 µm). Chl: Chlorophyll. **b**,
662 Co-immunoprecipitation (Co-IP) analyses using *N. benthamiana* leaves transiently
663 coexpressing LSD1-GFP and GLK1-Myc (or GLK2-Myc).

664 **Figure 2. LSD1 interacts with GLK1 and GLK2 *in vitro*.** **a,b**, Left panels: Gel
665 filtration profiles of LSD1, GLK1, and LSD1-GLK1 complex (**a**) and LSD1, GLK2,
666 and LSD1-GLK2 complex (**b**). A₂₈₀(mAU), micro-ultraviolet absorbance at the
667 wavelength of 280 nm. Right panels: Coomassie blue staining of the peak fractions
668 following SDS-PAGE. Numbers on top of SDS-PAGE panels indicate elution volume
669 (ml). M, molecular weight ladder (kDa).

670 **Figure 3. The conserved C-terminal region of GLK2 is required to interact with**
671 **LSD1 *in vitro*.** **a**, Schematic diagram of the predicted domain structure of GLK1 and
672 GLK2 along with truncated variants of the GLK2 protein (GLK2²⁴⁷⁻³⁸⁶ and
673 GLK2²⁹⁶⁻³⁸⁶). The DBD and GCT-box represent the DNA-binding domain and the
674 conserved C-terminal domain of GLK2, respectively. The light grey box (residues
675 247-265) between the DBD and GCT-box is a small conserved region in the GLK2
676 proteins. **b**, Gel filtration assays showing the *in vitro* interaction between LSD1 and
677 the truncated variants of GLK2 expressed in *E. coli*. Left panel: Gel filtration profiles
678 of LSD1, truncated variants of GLK2 and complex of LSD1 with the truncated
679 variants of GLK2. This experiment was repeated twice with similar results.
680 A₂₈₀(mAU), micro-ultraviolet absorbance at the wavelength of 280 nm. Right panel:
681 Coomassie blue staining of the peak fractions following SDS-PAGE, the numbers on
682 top of SDS-PAGE panels indicate elution volume (ml). M, molecular weight ladder
683 (kDa).

684 **Figure 4. The conserved C-terminal region of GLKs is necessary to interact with**

685 **LSD1 in vivo. a**, Subcellular localizations of intact GLKs (GLK1-GFP and
686 GLK2-GFP) and C-terminal-deleted (including the small conserved region shown in
687 Fig. 3a) GLKs (GLK1 Δ CT-GFP and GLK2 Δ CT-GFP) fused with GFP upon transient
688 expression in *N. benthamiana* leaves. Chl: chlorophyll. **b**, Bimolecular fluorescence
689 complementation (BiFC) assays. The intact and C-terminal-deleted GLKs fused with
690 the C-terminal part of YFP (YFP^C) were individually coexpressed with the N-terminal
691 part YFP (YFP^N) of the YFP^N-tagged LSD1 in *N. benthamiana* leaves. The
692 combination of LSD1-YFP^N+YFP^C was used as a negative control. DAPI was used to
693 stain the nucleus. All images in **a,b** were taken under the same scale (scale bars: 10
694 μ m). **c**, Co-IP analyses using *N. benthamiana* leaves transiently coexpressing
695 LSD1-GFP with GLK1-Myc, GLK1 Δ CT-Myc, GLK2-Myc, or GLK2 Δ CT-Myc.

696 **Figure 5. Loss of function of LSD1 leads to the upregulation GLK target genes. a**,
697 Venn diagram showing the numbers of uncommon and overlapped genes between
698 upregulated genes (395) in 17-d-old *lsd1*⁹ and downregulated genes (936) in *glk1*
699 *glk2*²³. **b**, Gene Ontology (GO) enrichment analysis towards the biological process of
700 the overlapped genes in **a**. The GO enrichment analysis was performed as described in
701 Methods. **c**, The relative expression levels of GLK1 and GLK2 target genes (*LHCBs*
702 and *LHCA1*) along with *LSD1* were examined in 17-d-old CL-grown plants of
703 wild-type (WT), *glk1 glk2*, and transgenic WT plants overexpressing GFP-tagged
704 LSD1 under the control of the 35S promoter (oxLSD1-GFP) using RT-qPCR. *ACT2*
705 was used as an internal standard for the analyses. The value represents means $\pm$
706 standard deviation (SD) (n=3). Lowercase letters indicate statistically significant
707 differences between mean values ($P < 0.05$, one-way ANOVA with posthoc Tukey's
708 HSD test). **d**, ChIP-qPCR results showing the effect of LSD1 overexpression on
709 GLK1 binding to the promoter regions of its target genes (*LHCB1.4*, *LHCB3*, and
710 *LHCB6*). 4xMyc-fused GLK1 (GLK1-Myc) was transiently expressed with (+ LSD1)
711 or without LSD1-RFP (– LSD1) in Arabidopsis leaf protoplasts isolated from *lsd1*
712 *glk1 glk2* triple mutant. The enrichment value was normalized to the input sample and
713 represents means $\pm$ SD from two independent ChIP assays. Asterisks denote
714 statistically significant differences by Student's *t*-test ($P < 0.01$) from the value of –
715 LSD1.

716 **Figure 6. *lsd1* mutation leads to the transcriptional upregulation of chlorophyll**
717 **synthesis genes. a**, A schematic representation of the chlorophyll synthesis pathway.

718 **b**, The expression levels of chlorophyll synthesis genes represented in **a** were
 719 obtained from our previous study⁹, and the relative transcript levels of chlorophyll
 720 synthesis genes in 17-d-old CL-grown *lsd1* mutants compared to wild type (WT) are
 721 represented. Error bars indicate SD (n=3). Asterisks indicate statistically significant
 722 differences (**P* < 0.05; ***P* < 0.01) in the *lsd1* mutant determined by Student's *t*-test
 723 relative to wild type. ND: non-detected. Red triangles in **a,b** indicate the genes that
 724 are significantly upregulated in the *lsd1* mutant compared to WT. **c**, The levels of the
 725 δ-ALA synthesis rate under CL. The rate of ALA synthesis was measured in 16-d-old
 726 plants of WT, *flu*, *lsd1*, and *lsd1 flu* grown under CL. **d**, Relative levels of
 727 protochlorophyllide (Pchl_{ide}) in 10-d-old plants of WT, *flu*, *lsd1*, and *lsd1 flu* grown
 728 under CL and then transferred to the dark for 8 hours. Values in **c,d** represent means ±
 729 SD (n=3). Lowercase letters indicate significant differences between the indicated
 730 genotypes (*P* < 0.05, one-way ANOVA with posthoc Tukey's HSD test).

731 **Figure 7. SA-induced SIB1 interferes with LSD1-GLK interaction.** **a**, The effect
 732 of the SA-induced SIB1 on the LSD1-GLK1 interaction. For Co-IP analyses,
 733 *35S:LSD1-GFP* and *35S:GLK1-Myc* were transiently coexpressed in Arabidopsis leaf
 734 protoplasts. The protoplasts were treated with either mock or 0.2 mM SA for 5 hours.
 735 **b**, The dose-dependent impact of SIB1 on the LSD1-GLK1 interaction. As indicated,
 736 *35S:LSD1-GFP* and *35S:GLK1-Myc* were coexpressed in Arabidopsis leaf protoplasts
 737 isolated from WT plants along with different amounts (10 µg, 20 µg, or 40 µg) of a
 738 plasmid containing either free *35S:RFP* or *35S:SIB1-RFP*. The subsequent Co-IP and
 739 immunoblot results are shown. Three independent experiments were conducted with
 740 similar results, and representative results are shown in **a,b**. **c**, The signal intensity of
 741 eluted GLK1-Myc (from triplicate immunoblots in **b**) versus its input signal was
 742 quantified using the ImageJ software. Data are means ± SD (n=3). Asterisks denote
 743 statistically significant differences by Student's *t*-test (**P* < 0.05, ***P* < 0.01, NS: not
 744 significant). **d**, Proposed model elucidates that SA-induced SIB1 antagonistically
 745 regulates LSD1-GLK interactions to reinforce the expression of PhANGs such as
 746 LHCBS and chlorophyll synthesis enzymes. The heightened expression of PhANGs
 747 eventually results in the enhanced generation of ¹O₂, activating EX1-mediated RS
 748 (contributing to cell death)⁹. On the other hand, the UPS primes the SIB1 proteolysis¹²,
 749 which would in turn facilitate LSD1-GLK interaction to avoid an excess expression of
 750 PhANGs and ¹O₂ accumulation. The counteractive regulation of the GLKs by SIB1

751 and LSD1 seems to be vital to maintain $^1\text{O}_2$ homeostasis in chloroplasts during and
752 after SA-increasing stress conditions.

Figure 1. LSD1 interacts with GLK1 and GLK2 *in vivo*. **a**, Bimolecular fluorescence complementation (BiFC) assays. The GLK1 and GLK2 fused with C-terminal part of YFP (YFP^C) were individually coexpressed with the N-terminal part of the YFP (YFP^N) tagged with LSD1 in *N. benthamiana* leaves. The combinations of LSD1-YFP^N+LSD1-YFP^C and LSD1-YFP^N+YFP^C were used as a positive and negative control, respectively. DAPI was used to stain the nucleus. All images were taken under the same scale (scale bars: 25 μm). Chl: Chlorophyll. **b**, Co-immunoprecipitation (Co-IP) analyses using *N. benthamiana* leaves transiently coexpressing LSD1-GFP and GLK1-Myc (or GLK2-Myc).

Figure 2. LSD1 interacts with GLK1 and GLK2 *in vitro*. **a,b**, Left panels: Gel filtration profiles of LSD1, GLK1, and LSD1-GLK1 complex (**a**) and LSD1, GLK2, and LSD1-GLK2 complex (**b**). A_{280} (mAU), micro-ultraviolet absorbance at the wavelength of 280 nm. Right panels: Coomassie blue staining of the peak fractions following SDS-PAGE. Numbers on top of SDS-PAGE panels indicate elution volume (ml). M, molecular weight ladder (kDa).

Figure 3. The conserved C-terminal region of GLK2 is required to interact with LSD1 *in vitro*. **a**, Schematic diagram of the predicted domain structure of GLK1 and GLK2 along with truncated variants of the GLK2 protein (GLK2₂₄₇₋₃₈₆ and GLK2₂₉₆₋₃₈₆). The DBD and GCT-box represent the DNA-binding domain and the conserved C-terminal domain of GLK2, respectively. The light grey box (residues 247-265) between the DBD and GCT-box is a small conserved region in the GLK2 proteins. **b**, Gel filtration assays showing the *in vitro* interaction between LSD1 and the truncated variants of GLK2 expressed in *E. coli*. Left panel: Gel filtration profiles of LSD1, truncated variants of GLK2 and complex of LSD1 with the truncated variants of GLK2. This experiment was repeated twice with similar results. A_{280} (mAU), micro-ultraviolet absorbance at the wavelength of 280 nm. Right panel: Coomassie blue staining of the peak fractions following SDS-PAGE, the numbers on top of SDS-PAGE panels indicate elution volume (ml). M, molecular weight ladder (kDa).

Figure 4. The conserved C-terminal region of GLKs is necessary to interact with LSD1 *in vivo*. **a**, Subcellular localizations of intact GLKs (GLK1-GFP and GLK2-GFP) and C-terminal-deleted (including the small conserved region shown in Fig. 3a) GLKs (GLK1 Δ CT-GFP and GLK2 Δ CT-GFP) fused with GFP upon transient expression in *N. benthamiana* leaves. Chl: chlorophyll. **b**, Bimolecular fluorescence complementation (BiFC) assays. The intact and C-terminal-deleted GLKs fused with the C-terminal part of YFP (YFP^C) were individually coexpressed with the N-terminal part YFP (YFP^N) of the YFP^N-tagged LSD1 in *N. benthamiana* leaves. The combination of LSD1-YFP^N+YFP^C was used as a negative control. DAPI was used to stain the nucleus. All images in **a,b** were taken under the same scale (scale bars: 10 μ m). **c**, Co-IP analyses using *N. benthamiana* leaves transiently coexpressing LSD1-GFP with GLK1-Myc, GLK1 Δ CT-Myc, GLK2-Myc, or GLK2 Δ CT-Myc.

Figure 5. Loss of function of LSD1 leads to the upregulation GLK target genes. **a**, Venn diagram showing the numbers of uncommon and overlapped genes between upregulated genes (395) in 17-d-old *lsd1*⁹ and downregulated genes (936) in *glk1 glk2*²³. **b**, Gene Ontology (GO) enrichment analysis towards the biological process of the overlapped genes in **a**. The GO enrichment analysis was performed as described in Methods. **c**, The relative expression levels of GLK1 and GLK2 target genes (*LHCBs* and *LHCA1*) along with *LSD1* were examined in 17-d-old CL-grown plants of wild-type (WT), *glk1 glk2*, and transgenic WT plants overexpressing GFP-tagged *LSD1* under the control of the 35S promoter (*oxLSD1*-GFP) using RT-qPCR. *ACT2* was used as an internal standard for the analyses. The value represents means $\pm$ standard deviation (SD) ($n=3$). Lowercase letters indicate statistically significant differences between mean values ($P < 0.05$, one-way ANOVA with posthoc Tukey's HSD test). **d**, ChIP-qPCR results showing the effect of *LSD1* overexpression on GLK1 binding to the promoter regions of its target genes (*LHCB1.4*, *LHCB3*, and *LHCB6*). 4xMyc-fused GLK1 (GLK1-Myc) was transiently expressed with (+ *LSD1*) or without *LSD1*-RFP (– *LSD1*) in Arabidopsis leaf protoplasts isolated from *lsd1 glk1 glk2* triple mutant. The enrichment value was normalized to the input sample and represents means $\pm$ SD from two independent ChIP assays. Asterisks denote statistically significant differences by Student's *t*-test ($P < 0.01$) from the value of – *LSD1*.

Figure 6. *lsd1* mutation leads to the transcriptional upregulation of chlorophyll synthesis genes. a, A schematic representation of the chlorophyll synthesis pathway. **b**, The expression levels of chlorophyll synthesis genes represented in **a** were obtained from our previous study⁹, and the relative transcript levels of chlorophyll synthesis genes in 17-d-old CL-grown *lsd1* mutants compared to wild type (WT) are represented. Error bars indicate SD (n=3). Asterisks indicate statistically significant differences (**P* < 0.05; ***P* < 0.01) in the *lsd1* mutant determined by Student's *t*-test relative to wild type. ND: non-detected. Red triangles in **a,b** indicate the genes that are significantly upregulated in the *lsd1* mutant compared to WT. **c**, The levels of the δ -ALA synthesis rate under CL. The rate of ALA synthesis was measured in 16-d-old plants of WT, *flu*, *lsd1*, and *lsd1 flu* grown under CL. **d**, Relative levels of protochlorophyllide (Pchlde) in 10-d-old plants of WT, *flu*, *lsd1*, and *lsd1 flu* grown under CL and then transferred to the dark for 8 hours. Values in **c,d** represent means $\pm$ SD (n=3). Lowercase letters indicate significant differences between the indicated genotypes (*P* < 0.05, one-way ANOVA with posthoc Tukey's HSD test).

Figure 7. SA-induced SIB1 interferes with LSD1-GLK interaction. **a**, The effect of the SA-induced SIB1 on the LSD1-GLK1 interaction. For Co-IP analyses, *35S:LSD1-GFP* and *35S:GLK1-Myc* were transiently coexpressed in Arabidopsis leaf protoplasts. The protoplasts were treated with either mock or 0.2 mM SA for 5 hours. **b**, The dose-dependent impact of SIB1 on the LSD1-GLK1 interaction. As indicated, *35S:LSD1-GFP* and *35S:GLK1-Myc* were coexpressed in Arabidopsis leaf protoplasts isolated from WT plants along with different amounts (10 µg, 20 µg, or 40 µg) of a plasmid containing either free *35S:RFP* or *35S:SIB1-RFP*. The subsequent Co-IP and immunoblot results are shown. Three independent experiments were conducted with similar results, and representative results are shown in **a,b**. **c**, The signal intensity of eluted GLK1-Myc (from triplicate immunoblots in **b**) versus its input signal was quantified using the ImageJ software. Data are means $\pm$ SD ($n=3$). Asterisks denote statistically significant differences by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, NS: not significant). **d**, Proposed model elucidates that SA-induced SIB1 antagonistically regulates LSD1-GLK interactions to reinforce the expression of PhANGs such as LHCBS and chlorophyll synthesis enzymes. The heightened expression of PhANGs eventually results in the enhanced generation of 1O_2 , activating EX1-mediated RS (contributing to cell death)⁹. On the other hand, the UPS primes the SIB1 proteolysis¹², which would in turn facilitate LSD1-GLK interaction to avoid an excess expression of PhANGs and 1O_2 accumulation. The counteractive regulation of the GLKs by SIB1 and LSD1 seems to be vital to maintain 1O_2 homeostasis in chloroplasts during and after SA-increasing stress conditions.