

1 **SIZ1-mediated SUMOylation of ROS1 Enhances Its Stability and Positively**  
2 **Regulates Active DNA Demethylation in *Arabidopsis***

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14

15 **Running title:** SIZ1 Promotes ROS1-mediated DNA Demethylation

16

17 **Short Summary:** The 5-methylcytosine DNA glycosylase/lyase REPRESSOR OF  
18 SILENCING 1 (ROS1) is indispensable for proper DNA methylation landscape in  
19 *Arabidopsis*. Whether and how the stability of ROS1 may be regulated by  
20 post-translational modifications is unknown. Here, we show that SIZ1-mediated  
21 SUMOylation of ROS1 enhances its stability and positively regulates active DNA  
22 demethylation.

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## 31 **Abstract**

32 The 5-methylcytosine DNA glycosylase/lyase REPRESSOR OF SILENCING 1  
33 (ROS1)-mediated active DNA demethylation is critical for shaping the genomic DNA  
34 methylation landscape in *Arabidopsis*. Whether and how the stability of ROS1 may be  
35 regulated by post-translational modifications is unknown. Using a  
36 methylation-sensitive PCR (CHOP-PCR)-based forward genetic screen for  
37 *Arabidopsis* DNA hypermethylation mutants, we identified the SUMO E3 ligase SIZ1  
38 as a critical regulator of active DNA demethylation. Dysfunction of SIZ1 leads to  
39 hyper-methylation at approximately one thousand genomic regions. SIZ1 physically  
40 interacts with ROS1 and mediates the SUMOylation of ROS1. The SUMOylation of  
41 ROS1 is reduced in *siz1* mutant plants. Compared to that in wild type plants, the  
42 protein level of ROS1 is significantly decreased, even though there is an increased  
43 level of *ROS1* transcripts in *siz1* mutant plants. Our results suggest that SIZ1  
44 positively regulates active DNA demethylation by promoting the stability of ROS1  
45 protein through SUMOylation.

46

## 47 **Introduction**

48 As an important and conserved epigenetic mark, 5-methylcytosine DNA methylation  
49 takes part in various biological processes in plants and animals (Chang et al., 2020;  
50 Law and Jacobsen, 2010; Liu and Lang, 2020; Scott and Spielman, 2004; Zhang et al.,  
51 2018). In plants, DNA methylation occurs in all sequence contexts (CG, CHG, and  
52 CHH, H represents for A, T and G) which are established *de novo* via the  
53 RNA-directed DNA methylation (RdDM) pathway and maintained by specific  
54 mechanisms according to the sequence context. MET1 and CMT3 are responsible for  
55 maintaining DNA methylation at symmetric CG and CHG contexts, respectively (Law  
56 and Jacobsen, 2010). The CHH methylation is maintained by DRM2 through the  
57 RdDM pathway and by CMT2 (Zemach *et al.*, 2013). DNA methylation is reversible  
58 and is determined by both methylation and demethylation processes (Zhu, 2009). The  
59 *Arabidopsis* 5-methylcytosine DNA glycosylase/lyase ROS1 is critical for pruning

60 DNA methylation to keep a proper DNA methylation pattern genome-wide (Gong *et*  
61 *al.*, 2002; Qian *et al.*, 2012). ROS1 is recruited to specific genomic loci by the  
62 cooperation of the Increased DNA Methylation (IDM) and SWR1 complex (Lang *et*  
63 *al.*, 2015; Li *et al.*, 2015; Li *et al.*, 2012; Nie *et al.*, 2019; Qian *et al.*, 2012; Wang *et*  
64 *al.*, 2015). MET18, a cytosolic iron-sulfur assembly (CIA) pathway component, was  
65 identified as an important regulator for the enzymatic activity of ROS1 (Duan *et al.*,  
66 2015; Wang *et al.*, 2016). Little is known about whether and how ROS1 may be  
67 regulated by post-translational modifications.

68

69 Mechanistically similar to ubiquitination, SUMOylation occurs through an  
70 ATP-dependent enzyme cascade, including heterodimeric E1 activating enzymes  
71 (SAE1/SAE2), E2 conjugating enzyme (SCE1), E3 ligase and SUMO proteases  
72 SENPs/Ulps (Augustine and Vierstra, 2018; Mukhopadhyay and Dasso, 2007; Seeler  
73 and Dejean, 2003). The SUMO E3 ligase can facilitate SUMO conjugation by E2 to  
74 the substrates and increase the substrate specificity (Gareau and Lima, 2010; Johnson,  
75 2004). In *Arabidopsis*, the SUMO E3 ligase SIZ1 contains five domains including the  
76 SAP domain for nuclei acid binding, PHD domain for selecting targets and  
77 recognizing H3K4me3, PINIT motif required for the E3 ligase activity, SP-RING zinc  
78 finger domain for the E3 ligase activity and localization of SIZ1, and SXS motif for  
79 SUMO interaction (Cheong *et al.*, 2009; Garcia-Dominguez *et al.*, 2008; Miura *et al.*,  
80 2007a; Miura *et al.*, 2020). Dysfunction of SIZ1 is reported to affect abiotic and biotic  
81 stress responses, phosphate starvation responses, flowering time and  
82 photomorphogenesis (Lin *et al.*, 2016; Lois *et al.*, 2003; Mazur *et al.*, 2019; Miura *et*  
83 *al.*, 2007a; Miura *et al.*, 2007b; Miura *et al.*, 2009; Miura *et al.*, 2005).

84

85 Here, we show that SIZ1 positively regulates active DNA demethylation mainly  
86 through a ROS1-dependent pathway. Mutation of *SIZ1* leads to a genome-wide  
87 hyper-methylation phenotype similar to that in *ros1* mutants. We found that SIZ1  
88 directly interacts with ROS1 and facilitates the SUMO modification of ROS1.  
89 Dysfunction of SIZ1 causes defects in ROS1 SUMOylation and a reduction in ROS1

90 protein level. Our study reveals an important connection between SUMOylation and  
91 active DNA demethylation in plants.

92

## 93 **Results and Discussion**

### 94 **SIZ1 positively regulates active DNA demethylation independently of SA**

95 To identify candidate genes involved in active DNA demethylation, we screened for  
96 T-DNA insertion mutants of *Arabidopsis thaliana* by CHOP-PCR based on DNA  
97 hyper-methylation phenotype at the 3' region of *At1g26400* as previously described  
98 (Qian *et al.*, 2012). Two mutants bearing T-DNA insertion in the SUMO E3 ligase  
99 gene *SIZ1* (SALK\_065397/*siz1-2* and SALK\_034008/*siz1-3*) showed a  
100 hyper-methylation phenotype (Fig. 1A and S1A). Locus-specific bisulfite sequencing  
101 result of the 3' region of *At1g26400* indicated that in *ros1-4*, *siz1-2* and *siz1-3*, the  
102 methylation levels increased in all sequence contexts (i.e. CG, CHG and CHH),  
103 although the increase in non-CG methylation is not as pronounced as in CG  
104 methylation (Fig. 1B). CHOP-PCR tests on another three ROS1 target loci  
105 (*At1g26390*, *At1g26410* and *At4g18650*) were performed and all were found  
106 hyper-methylated in *siz1* mutants (Fig. 1C). Moreover, methylation analysis of the  
107 *ros1-4siz1-2* double mutant revealed that the hyper-methylation phenotypes of *siz1-2*  
108 and *ros1-4* were not additive (Fig. 1B and 1D), suggesting that SIZ1 and ROS1 may  
109 function in the same genetic pathway for active DNA demethylation.

110

111 Specific domains of SIZ1 control unique responses to different environmental stimuli  
112 (Cheong *et al.*, 2009). To examine the function of the various domains of SIZ1 in  
113 regulating active DNA demethylation, we performed CHOP-PCR assay to analyze the  
114 *SIZ1*<sup>WT</sup>, *SIZ1*<sup>sap</sup>, *SIZ1*<sup>phd</sup>, *SIZ1*<sup>pinit</sup>, *SIZ1*<sup>sp-ring</sup> and *SIZ1*<sup>sxs</sup> plants and found that only  
115 expression of the wild type SIZ1 could rescue the hyper-methylation phenotype of  
116 *siz1-2* (Fig. S1B), demonstrating that intact SIZ1 protein is important for regulating  
117 DNA demethylation at the 3' region of *At1g26400*. Dysfunction of SIZ1 leads to an  
118 obvious dwarf phenotype due to the elevated salicylic acid (SA) level (Lee *et al.*,  
119 2007). We introduced the *nahG* gene encoding a bacterial salicylate hydroxylase into

120 *siz1-2* mutant plants to examine the effect of over-accumulated SA on DNA  
121 methylation. The expression of *nahG* rescued the dwarf phenotype of *siz1-2* but did  
122 not affect the DNA hyper-methylation phenotype (Fig. S2A and S2B), indicating that  
123 the hyper-methylation phenotype of *siz1-2* is not due to the elevated SA.

124

125 The indispensable role of SUMOylation in plant viability makes it difficult to analyze  
126 knock-out mutants of key genes in the SUMOylation pathway (Saracco *et al.*, 2007).  
127 Over-expressing a mutated form of SCE1 containing the C94S substitution results in a  
128 dominant-negative effect, which impairs the SUMOylation process as previously  
129 reported (Tomanov *et al.*, 2013). We generated SCE1(WT/C94S)-Flag  
130 over-expression plants (Fig. S3A), and found that plants over-expressing SCE1(C94S)  
131 showed a dwarf phenotype like *siz1* mutants (Fig. S3B). Only SCE1(C94S)-Flag  
132 over-expressing plants showed DNA hyper-methylation at the 3' region of *At1g26400*  
133 similarly to *siz1-2* mutants (Fig. S3C), demonstrating that proper SUMOylation is  
134 important for active DNA demethylation.

135

### 136 **SIZ1 affects DNA methylation at over a thousand genomic regions**

137 According to whole genome bisulfite sequencing data, 1,040 hyper-methylated DMRs  
138 (hyper-DMRs) and 183 hypo-methylated DMRs (hypo-DMRs) were identified in  
139 *siz1-2* mutant plants (Fig. 2A, Table S1 and Table S2). In accordance with  
140 locus-specific bisulfite sequencing results, whole genome bisulfite sequencing  
141 showed that *At1g26390*, *At1g26400*, *At1g26410* and *At4g18650* were all  
142 hyper-methylated compared to Col-0 (Fig. S4A), although only *At1g26410* was  
143 counted as a hyper-DMR by the stringent parameters used in this study. The  
144 hyper-DMRs in *siz1-2*, *ros1-4* and *rdd* (a triple mutant defective in *ROS1* and its  
145 paralogs *DML2* and *DML3*) mutants were distributed similarly in all five  
146 chromosomes (Fig. S4B and S4C). Analysis of hyper-DMRs in different genomic  
147 regions indicated that similar to that in *ros1-4*, ~ 26% of hyper-DMRs in *siz1-2* were  
148 located in TE regions (Fig. S4D). Nine of the hyper-DMRs shared by *siz1* and *ros1*  
149 mutants were validated by CHOP-PCR (Fig. S5A and S5B).

150 Approximately 70% of the hyper-DMRs identified in *siz1-2* mutant plants overlapped  
151 with those in *ros1-4* and *rdd* mutant plants (Fig. 2A, 2B and Table S1). The DNA  
152 methylation level of the overlapping hyper-DMRs was increased in all cytosine  
153 contexts (Fig. 2B). However, the DNA methylation level of *ros1-4* specific  
154 hyper-DMRs was not increased in *siz1-2*, suggesting that SIZ1 affects a subset of  
155 ROS1 target loci. DNA methylation change is usually accompanied with perturbation  
156 of nearby genes' expression (Harris et al., 2018; Liu and Lang, 2020; Yang et al., 2019;  
157 Zhao et al., 2019). To investigate whether the increased DNA methylation in *siz1-2*  
158 contributes to gene expression regulation, we analyzed 14 genes near the  
159 hyper-DMRs by RT-qPCR and found that six of the genes showed decreased  
160 transcript levels in *siz1* and *ros1* mutants (Fig. S6). These results suggested that SIZ1  
161 may regulate the expression of these genes by affecting DNA methylation.

162 Among the 183 hypo-DMRs identified in *siz1-2* mutant plants, 179 (97.8%), 56  
163 (30.6%), and 105 (57.4%) overlapped with hypo-DMRs in *met1*, *cmt3*, and *nrdp1*,  
164 respectively (Fig. S7 A-C and Table S2). For the overlapping hypo-DMRs of *siz1-2*  
165 and *met1*, their DNA methylation levels were decreased in all three cytosine contexts  
166 including CG, CHG and CHH (Fig. S7A). The DNA methylation levels of the  
167 overlapping hypo-DMRs between *siz1-2* and *cmt3* and between *siz1-2* and *nrdp1* were  
168 reduced mainly at CHG and CHH contexts, respectively (Fig. S7B and S7C). Even  
169 for the *siz1*-specific hypo-DMRs, the CHG and CHH DNA methylation levels were  
170 also decreased in *cmt3* and *nrdp1* (Fig. S7B and S7C). Heatmap analysis showed that  
171 the DNA methylation levels of most *siz1* hypo-DMRs were also reduced in *met1*,  
172 while the majority of non-CG methylation at *siz1* hypo-DMRs was decreased in *cmt3*  
173 and *nrdp1* (Fig. S7D). These results indicated that the DNA methylation at the *siz1-2*  
174 hypo-DMRs was mainly contributed by MET1, although CMT3 and RdDM may also  
175 contribute to the non-CG methylation in these regions. The potential contribution of  
176 CMT3 is in line with previous report of positive regulation of CMT3 activity by  
177 SUMOylation that is dependent on SIZ1 (Kim et al., 2015).

178

179 **SIZ1 physically interacts with ROS1**

180 To examine if SIZ1 physically interacts with ROS1, yeast two-hybrid assays were  
181 performed. Only yeast cells carrying both SIZ1-AD and ROS1-BD grew well on the  
182 SD-Leu/-Trp/-His/-Ade media and turned blue with the supplement of X- $\beta$ -Gal (Fig.  
183 3A), indicating that SIZ1 interacts with ROS1 in yeast. The direct interaction between  
184 SIZ1 and ROS1 was confirmed by split-LUC and bimolecular fluorescence  
185 complementation (BiFC) assays in *Nicotiana benthamiana* leaves. In the split-LUC  
186 assay, strong LUC signals were detected only when cLUC-SIZ1 and ROS1-nLUC  
187 were co-expressed (Fig. 3B). The BiFC results showed a YFP fluorescence signal in  
188 the nucleus of *Nicotiana benthamiana* cells co-expressing SIZ1-cYFP and  
189 ROS1-nYFP (Fig. 3C), suggesting that SIZ1 interacts with ROS1 in the nucleus.  
190 Additionally, HA-SIZ1 could be co-immunoprecipitated with ROS1-Flag (Fig. 3D)  
191 when they were co-expressed in *Arabidopsis* protoplasts, which further confirmed the  
192 interaction between SIZ1 and ROS1. These results showed that SIZ1 physically  
193 interacts with ROS1 in the nucleus.

194

### 195 **SIZ1 enhances the SUMOylation of ROS1 and stabilizes ROS1**

196 The interaction between SIZ1 and ROS1 further prompted us to test whether ROS1  
197 may be SUMOylated. Using the split-LUC assay, we found that only the combination  
198 of ROS1-nLUC or ROS1b-nLUC (510-1393 aa) with cLUC-SUMO1 showed strong  
199 LUC signals, indicating that ROS1 interacts with SUMO1 via ROS1 C-terminal  
200 region containing the DNA glycosylase and CTD domains (Fig. 4A). According to the  
201 *in vitro* SUMOylation assay described previously (Okada *et al.*, 2009) and  
202 SUMOylation site prediction (Fig. S8), ROS1c (1-290 aa, containing K133) and  
203 ROS1d (794-1063 aa, containing K806/K812/K851/K1051) fused with the T7-tag  
204 were generated. Immunoblot analysis detected a shift of protein band to higher  
205 molecular weight when ROS1d but not ROS1c was co-expressed with SUMO E1,  
206 SUMO E2 and the mature SUMO1GG (Fig. 4B). A similar result was obtained when  
207 T7-ROS1c/ROS1d was replaced with GST-ROS1c/ROS1d-Myc in the assay (Fig.  
208 S9A and S9B), suggesting that ROS1 can be SUMOylated *in vitro*, and the  
209 SUMOylation was mainly in the DNA glycosylase domain.



210 An *in vivo* SUMOylation assay was performed to confirm the SUMOylation of ROS1.  
211 A shift of protein band to higher molecular weight was detected when ROS1-Flag or  
212 ROS1b-Flag was co-expressed with HA-SUMO1 in Col-0 protoplasts. The higher  
213 molecular weight band was confirmed to contain both ROS1 and SUMO1 by  
214 LC-MS/MS analysis (Fig. 4C and S10). The band corresponding to SUMO1-modified  
215 ROS1 was substantially weaker when the proteins were expressed in *siz1-2*  
216 protoplasts (Fig. 4D), indicating that SIZ1 enhances the SUMO1 modification of  
217 ROS1. The lysine to arginine substitution can block the SUMOylation on substrate  
218 proteins (Gostissa *et al.*, 1999), so we mutated 5 predicted SUMOylation sites to  
219 generate ROS1(5K/Rs)-Flag and co-expressed the mutant protein with HA-SUMO1  
220 in Col-0 protoplasts. The SUMO1 modification of ROS1 was blocked by these 5  
221 lysine to arginine substitutions (Fig. 4D), demonstrating that ROS1 is SUMOylated at  
222 one or more of these 5 predicted lysine residues.

223 Finally, we examined the effect of SIZ1-mediated SUMOylation on ROS1 by  
224 analyzing the protein level of ROS1 in *siz1-2* mutant. An *ROS1-GFP/siz1-2* line was  
225 obtained by crossing *siz1-2* to the *GFP* knock-in line *ROS1-GFP/Col-0* generated in a  
226 previous study (Miki *et al.*, 2018). Although *ROS1* transcript level significantly  
227 increased in *siz1-2* mutant (Fig. S11A and S11B), the immunoblotting result showed  
228 that the ROS1-GFP protein was substantially decreased in *siz1-2* mutant plants (Fig.  
229 4E and 4F). Together, these results support that SIZ1 enhances the stability of ROS1  
230 by facilitating the SUMOylation of ROS1.

231 The role of SUMO modification in active DNA demethylation has been reported in  
232 mammals (McLaughlin *et al.*, 2016; Steinacher *et al.*, 2019; Waters *et al.*, 1999). In  
233 *Arabidopsis*, some components of the RdDM pathway and base excision repair (BER)  
234 pathway were found to be SUMOylated or interact with SUMOs (Elrouby *et al.*, 2013;  
235 Kim *et al.*, 2015; Miller *et al.*, 2010; Rytz *et al.*, 2018). In this study, we showed that  
236 the SUMO E3 ligase SIZ1 positively regulates active DNA demethylation by  
237 mediating SUMOylation of ROS1, leading to enhanced ROS1 stability (Fig. 4A-4D,  
238 S8-S10). Additionally, SIZ1 may regulate DNA methylation by facilitating the  
239 SUMOylation of DNA methyltransferases such as CMT3 and other methylation



240 regulators, which are possibly responsible for the hypo-DMRs in *siz1* mutant plants  
241 (Fig. 2A and S7) (Augustine and Vierstra, 2018; Kim et al., 2015).

242 In mammals, the DNA glycosylase TDG is SUMOylated to enhance its enzymatic  
243 turnover, leading to more efficient active DNA demethylation (McLaughlin *et al.*,  
244 2016; Waters *et al.*, 1999). Here, we showed that the important DNA glycosylase  
245 ROS1 in *Arabidopsis* can also be SUMOylated and this is facilitated by SUMO E3  
246 ligase SIZ1 (Fig. 4A-4D, S8-S10). The accumulation of ROS1 protein is reduced  
247 while *ROS1* transcripts are increased in *siz1* mutant plants (Fig. 4E, 4F and S11),  
248 which may be due to feedback regulation through the DNA methylation monitoring  
249 sequence at the *ROS1* gene promoter as reported in previous studies (Lei et al., 2015;  
250 Qian et al., 2014; Xiao et al., 2019; Zheng et al., 2008). Only a certain subset of  
251 ROS1 target loci was affected in *siz1* mutant plants, which may be caused by the  
252 distinct requirements of ROS1 function at different genomic regions. Since the  
253 possible SUMOylation sites are located in the DNA glycosylase domain that is critical  
254 for 5-methylcytosine excision activity of the 5-methylcytosine DNA glycosylases  
255 (Gehring et al., 2006; Mok et al., 2010; Ortega-Galisteo et al., 2008), the role of  
256 SUMOylation on the enzymatic activity or turnover of ROS1 needs to be tested once  
257 the precise SUMOylated residues are determined. In addition, it will be interesting to  
258 study the role of SIZ1-mediated ROS1 SUMOylation in regulating ABA responses,  
259 considering the similar DNA hyper-methylation in the promoter region of *DOLG4*  
260 displayed by both the *siz1* and *ros1-4* mutants (At4g18650, Fig. 1C and S4A) (Zhu et  
261 al., 2018). Since ROS1 has been suggested to be modified by ubiquitination, whether  
262 SUMOylation of ROS1 competitively affects the ubiquitination of ROS1 to reduce its  
263 degradation remains to be determined in the future (Hay, 2005; Maor et al., 2007;  
264 Miura et al., 2007b; Ulrich, 2005).

265

## 266 **Materials and Methods**

### 267 **Plant materials and CHOP-PCR-based screening**

268 The *Arabidopsis siz1-2* (SALK\_065397) and *siz1-3* (SALK\_034008) mutant lines  
269 were obtained from the Arabidopsis Biological Resource Center (ABRC,

270 <http://www.arabidopsis.org>). The coding sequence of *SCE1*(WT) or *SCE1*(C94S)  
271 were cloned into pCAMBIA1305 vector and introduced into Col-0 plants to generate  
272 *SCE1* (WT) and *SCE1* (C94S) over-expression lines. Seedlings were grown on 1/2  
273 Murashige and Skoog (MS) agar plates at 22 °C under 16 h light/8 h dark photoperiod.  
274 Plants including *Arabidopsis* and *Nicotiana benthamiana* were grown in soil at 22 °C  
275 under a 16 h light/8 h dark photoperiod. Screening for mutants was as described (Qian  
276 *et al.*, 2012). Primers are listed in Table S3.

277

### 278 **Locus-specific bisulfite sequencing**

279 Experiment was performed as described (Li et al., 2020). Primers used for PCR were  
280 listed in Table S3.

281

### 282 **Whole genome bisulfite sequencing and data analysis**

283 Fourteen-day-old seedlings were used for genomic DNA extraction using DNeasy  
284 Plant Kit (Qiagen) following the manufacturer's protocols. Bisulfite treatment, library  
285 construction, and deep sequencing were performed by the Genomics Core Facility at  
286 the Shanghai Center for Plant Stress Biology, China.

287 Adaptor and low-quality sequences ( $q < 20$ ) were trimmed to generate clean reads that  
288 were then mapped to the TAIR 10 genome using BSMAP (Bisulfite Sequence  
289 Mapping Program) and allowing two mismatches. DMRs were counted according to  
290 Qian et al. (2012) with some modifications. Briefly, the cytosines with  $\geq 4X$   
291 coverage were considered. DMCs were identified if the  $P$ -value calculated by  
292 two-tailed Fisher's Exact test was  $< 0.01$ . Genome was divided into 1kb regions, in  
293 which the number of DMCs was counted. A region with at least 5 DMCs was  
294 considered as an anchor region, of which the actual boundary was adjusted as the  
295 locations of the first DMC and last DMC. The anchor regions were combined into a  
296 larger region if the distance between two anchor regions was  $\leq 1\text{kb}$ . A DMR was  
297 reported if the larger region contained at least 7 DMCs.

298

### 299 **Yeast two-hybrid assay**

300 Yeast two-hybrid assay was carried out as described previously (Hong *et al.*, 2020).  
301 Briefly, the coding sequence of *ROS1* and *SIZ1* were cloned into pGBKT7 and  
302 pGADT7 (Clontech), respectively. Different combinations of bait and prey plasmids  
303 were co-transformed as indicated.

304

#### 305 **Split-LUC assay and Bimolecular fluorescence complementation (BiFC) assay**

306 The split-LUC assay and BiFC assay were performed as described (Hong *et al.*, 2020).  
307 Briefly, the full-length coding sequences of *ROS1*, *SIZ1* and *SUMO1* were amplified  
308 to generate cLUC-SIZ1, cLUC-SUMO1, ROS1-nLUC, SIZ1-p2YC and ROS1-p2YN  
309 constructs. The truncated ROS1a/ROS1b-nLUC constructs were generated from  
310 ROS1-nLUC using indicated primers (Table S3).

311

#### 312 **Co-Immunoprecipitation assay**

313 The full-length coding sequences of *ROS1* and *SIZ1* were cloned into pUC18-Flag  
314 and pUC18-HA vectors separately, then co-expressed in Col-0 protoplasts by  
315 polyethylene glycol (PEG400)-mediated transformation as previously described (Yoo  
316 *et al.*, 2007). The protoplasts were collected and lysed after an incubation for 20 h at  
317 22 °C . Fifty µl of anti-Flag mAb-Magnetic beads (MBL International) were added to  
318 the crude lysate supernatant and gently rotated for 2 h at 4 °C. The  
319 immunoprecipitated proteins were detected by Western-blotting using anti-HA  
320 antibody (Roche) and anti-Flag antibody (Sigma).

321

#### 322 **SUMOylation assay and mass spectrometry**

323 The *in vitro* SUMOylation assay was performed as previously described (Okada *et al.*,  
324 2009). The pET28a\_ ROS1c / ROS1d expressing N-terminal T7-tagged variant ROS1  
325 (ROS1c: 1-290 aa, ROS1d: 794-1063 aa) and pGEX4T1\_ROS1c / ROS1d expressing  
326 GST-ROS1c/ROS1d-Myc were generated. The SUMOylation was analyzed by  
327 Western-blotting using anti-T7 antibody (Abcam) or anti-Myc antibody (Millipore).

328

329 *In vivo* SUMOylation assay was conducted as described (Zheng *et al.*, 2012).The

330 coding sequences of *ROS1* or *SUMO1* were cloned into pUC18-Flag or pUC18-HA  
331 respectively and co-expressed in Col-0 or *siz1-2* protoplasts. The protoplasts were  
332 collected after 20 h of incubation at 22 °C. The proteins were immunoprecipitated by  
333 anti-Flag mAb-Magnetic beads (MBL International) detected by Western-blotting  
334 using anti-HA antibody (Roche) and anti-Flag antibody (Sigma).

335

336 For LC-MS/MS analysis, the anti-Flag mAb-Magnetic beads immunoprecipitated  
337 proteins were separated in SDS-PAGE gel followed by silver staining using Fast  
338 Silver Stain Kit (Beyotime). The bands of ROS1 and SUMOylated ROS1 were cut  
339 into pieces according to their molecular weights and used for LC-MS/MS analysis at  
340 BGI.

341

#### 342 **RNA extraction and Real-Time quantitative RT-PCR**

343 RNA extraction and gene transcripts level determination by RT-qPCR were  
344 performed using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co.,Ltd)  
345 according to (Hong *et al.*, 2020). *UBQ10* and *ACT2* were used as internal control. The  
346 primers used were listed in Table S3.

347

#### 348 **Accession numbers**

349 Sequence data from this article can be found in The Arabidopsis Information  
350 Resource (<http://www.arabidopsis.org/>) under the following accession numbers: *SIZ1*  
351 (At5g60410), *ROS1* (At2g36490), *SUMO1* (At4g26840), *SCE1* (At3g57870), *TUB8*  
352 (At5g23860), *UBQ10* (At4g05320), *ACT2* (At3g18780). The methylome data used in  
353 this study can be found in NCBI GEO by the following accession numbers:  
354 GSE152425 (*siz1-2*), GSE33071 (Qian *et al.*, 2012) (Col-0, *ros1-4* and *rdd*),  
355 GSE39901 (Stroud *et al.*, 2013) (*met1*, *cmt3* and *nprdl*).

356

#### 357 **Supplemental Items**

358 **Supplemental Figure 1.** Characterization of *siz1* mutants.

359 **Supplemental Figure 2.** Hyper-methylation caused by *siz1* mutation is not due to the

360 elevated SA.

361 **Supplemental Figure 3.** Plants over-expressing dominant-negative SUMO E2  
362 SCE1(C94S) show DNA hypermethylation at *At1g26400* loci.

363 **Supplemental Figure 4.** Analysis of DNA methylation pattern of *siz1-2* by whole  
364 genome bisulfite sequencing.

365 **Supplemental Figure 5.** Examples of hyper-methylated regions.

366 **Supplemental Figure 6.** DNA hypermethylation represses the expression of nearby  
367 genes in *siz1* mutants.

368 **Supplemental Figure 7.** Features of *siz1-2* hypo-DMRs.

369 **Supplemental Figure 8.** Predicted SUMOylation sites in ROS1.

370 **Supplemental Figure 9.** ROS1 is SUMOylated.

371 **Supplemental Figure 10.** Detection of SUMOylated ROS1 by LC-MS/MS.

372 **Supplemental Figure 11.** *ROS1* transcript level in the *siz1* mutants.

373 **Supplemental Table 1.** List of hyper-DMRs identified in *siz1-2*, *ros1-4* and *rdd*  
374 mutants.

375 **Supplemental Table 2.** List of hypo-DMRs of *siz1-2*, *met1*, *cmt3* and *nrdp1* mutants.

376 **Supplemental Table 3.** Primers used in this study.

377

### 378 **Author contributions**

379 J.-K.Z., X.K. and Y.-F.H conceived the research. X.K., Y.H., Y.-F.H., X.L. and Z.S.  
380 performed experiments. H.H. analyzed the methylome data. X.K., Y.H., Y.-F.H. and  
381 J.-K.Z. wrote the manuscript.

382

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387 transgenic plants of *SIZ1* carrying point mutations at specific domains (*SIZ1*<sup>sap</sup>,  
388 *SIZ1*<sup>phd</sup>, *SIZ1*<sup>pinit</sup>, *SIZ1*<sup>sp-ring</sup>, *SIZ1*<sup>sxs</sup>), Drs. Chao-Feng Huang and Jie Zhang for their  
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391

### 392 **Conflict of interest**

393 The authors declare no competing interests.

394

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570

## 571 **Figure legends**

### 572 **Figure 1. The *siz1* mutant plants show DNA hyper-methylation at multiple loci.**

573 (A) Analysis of DNA methylation status at the 3' region of *At1g26400* by  
574 methylation-sensitive PCR (CHOP-PCR). Compared to that in wild type Col-0 plants,  
575 the methylation levels increased in *ros1-4*, *siz1-2* and *siz1-3* mutants. Undigested  
576 genomic DNA was used as a control. (B) Analysis of DNA methylation status at the 3'  
577 region of *At1g26400* via locus-specific bisulfite sequencing. (C) Analysis of DNA  
578 methylation status at multiple loci by CHOP-PCR. Similar to those in *ros1-4* mutant,  
579 the methylation levels increased in *siz1-2* and *siz1-3* mutants. (D) Analysis of DNA  
580 methylation status at the indicated loci via locus-specific bisulfite sequencing.

581 See also Figures S1 and S2.

582

### 583 **Figure 2. Effect of *SIZ1* mutation on genome-wide DNA methylation.** (A)

584 Numbers of hyper/hypo-DMRs identified in *siz1-2*, *ros1-4* and *rdd* mutants by  
585 whole-genome bisulfite sequencing, and the overlaps in hyper-DMRs between  
586 different mutants. (B) Venn diagram displaying the numbers of hyper-DMRs that are  
587 overlapping or unique in *siz1-2* and *ros1-4*. Box plots showing the distribution of  
588 average DNA methylation levels in CG, CHG and CHH contexts that were calculated  
589 from the indicated overlapping or unique hyper-DMRs.

590 See also Figures S4, S5, S6 and Table S1-2.

591

### 592 **Figure 3. *SIZ1* physically interacts with *ROS1*.** (A) Yeast two-hybrid result

593 showing an interaction between *SIZ1* and *ROS1*. Full-length *SIZ1* and *ROS1* were  
594 fused to AD and BD, respectively. The different combination of recombinant plasmids

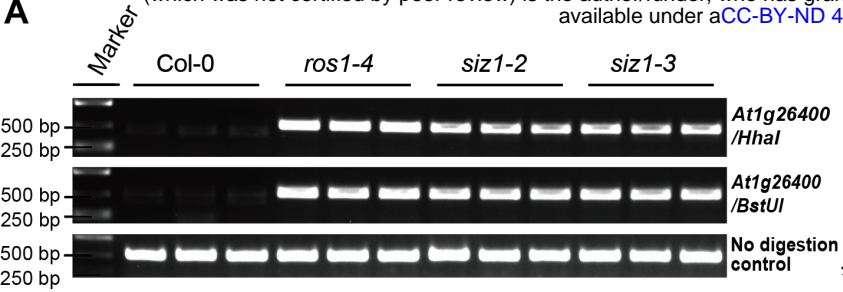
595 and empty vectors were co-transformed into yeast cells. AD, Gal4 activation domain;  
596 BD, Gal4 binding domain. (B) Split-LUC assay showing an interaction between SIZ1  
597 and ROS1 in *Nicotiana benthamiana* leaves. Firefly luciferase was fused with the  
598 coding sequences of *SIZ1* and *ROS1* at their N-terminus and C-terminus, respectively.  
599 (C) Bimolecular fluorescence complementation (BiFC) test indicating SIZ1  
600 interaction with ROS1 in *Nicotiana benthamiana* leaves in the nucleus. Full-length  
601 SIZ1 and ROS1 were fused to YFP at their C-terminus. Chlorophyll, the  
602 autofluorescence of chlorophyll; BF, bright field; Scale bars, 20  $\mu$ m. Inset, 4x  
603 magnification of boxed region. (D) Co-immunoprecipitation showing an association  
604 of SIZ1 with ROS1. HA-tagged SIZ1 and Flag-tagged ROS1 were expressed  
605 separately or co-expressed in *siz1-2* protoplasts. HA-SIZ1 and ROS1-Flag were  
606 detected by immuno-blotting using anti-HA and anti-Flag antibodies with crude lysate  
607 proteins and proteins immunoprecipitated via anti-Flag mAb-Magnetic beads.

608

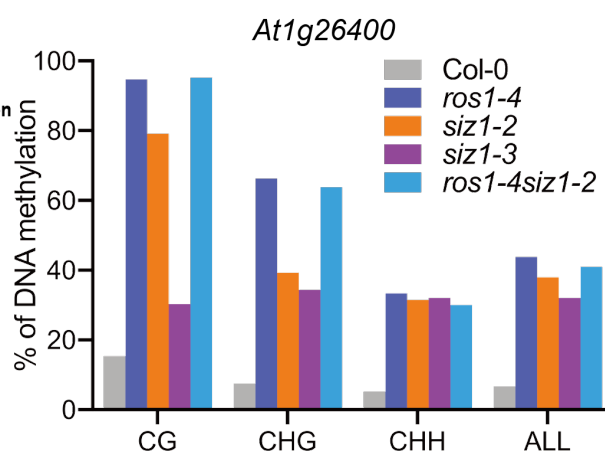
609 **Figure 4. SIZ1 mediated SUMOylation of ROS1 enhances its stability.** (A) The  
610 interaction of SUMO1 and ROS1 was tested using the split-LUC assay. The  
611 C-terminal end of firefly luciferase was fused to the N-terminus of SUMO1. ROS1  
612 was fused to the N-terminal end of firefly luciferase. The schematic diagram shows  
613 the full-length ROS1 and truncated ROS1 (ROS1a: 1-509 aa, ROS1b: 510-1393 aa).  
614 NTD, N-terminal domain; CTD, C-terminal domain. (B) SUMOylation of ROS1 was  
615 tested using the *Arabidopsis* SUMOylation pathway reconstitution system in *E. coli*.  
616 Different combinations of the three plasmids encoding *Arabidopsis* SUMOylation  
617 machinery proteins and truncated ROS1 indicated above each lane were  
618 co-transformed into *E. coli* BL21(DE3). Immuno-blotting was used to detect  
619 truncated ROS1 and its SUMOylated forms using anti-T7 antibodies in crude lysate  
620 proteins. SUMOylated ROS1 shifting to a larger molecular weight band was observed  
621 only in the lane using the mature wild type form of SUMO1 (referred to as  
622 SUMO1GG), but not in lanes using mutated form of SUMO1 (referred to as  
623 SUMO1AA). The schematic diagram indicates the truncated ROS1 (ROS1c: 1-290 aa  
624 containing K133), ROS1d: 794-1063 aa containing K806, K812, K851 and K1051).

625 (C) *In vivo* SUMOylation of ROS1. ROS1-Flag/ROS1b-Flag and HA-SUMO1 were  
626 co-expressed in Col-0 protoplasts. The SUMOylated ROS1 was detected via  
627 immuno-blotting using anti-HA antibodies in proteins immunoprecipitated by  
628 anti-Flag mAb-Magnetic beads. Expression of HA-SUMO1 with empty vector served  
629 as a negative control. (D) SUMOylation of ROS1 was reduced in *siz1-2*. ROS1-Flag  
630 (lane1) / ROS1(5K/Rs)-Flag (lane2) and HA-SUMO1 were co-expressed in Col-0  
631 protoplasts. ROS1-Flag and HA-SUMO1 were co-expressed in *siz1-2* protoplasts  
632 (lane3). SUMOylation in mutant ROS1 with the 5 predicted SUMOylation sites  
633 mutated by lysine to arginine substitutions (K133R/K806R/K812R/K851R/K1051R)  
634 was reduced (lane2). SUMOylation of ROS1 was substantially decreased in *siz1-2*  
635 compared to that in Col-0 (lane 3). (E) Immunodetection of ROS1 by an anti-GFP  
636 antibodies in GFP knock-in lines with GFP integrated in the endogenous *ROS1* locus  
637 in Col-0 and *siz1-2* background. Col-0 and *siz1-2* without the GFP served as negative  
638 controls. Actin was used as a loading control. The protein level of ROS1 relative to  
639 Actin shown above the lanes was quantified using Image Lab software (version 5.2.1,  
640 Bio-Rad). (F) Statistical analysis of the ROS1 protein levels in *siz1-2* mutant (n=3).  
641 \*\*\*  $P < 0.001$ , determined by Student's *t*-test.  
642 See also Figures S8, S9, S10 and S11.

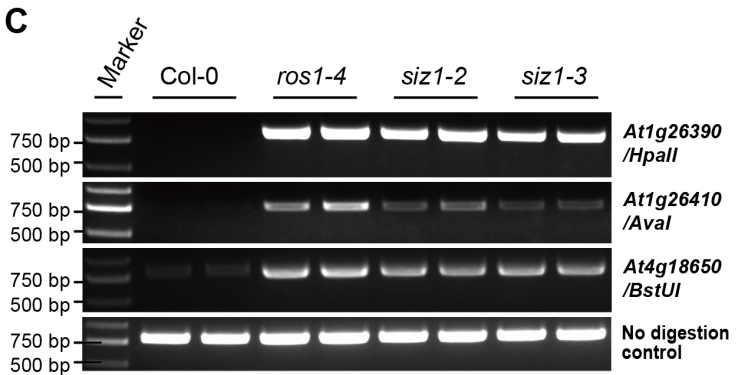
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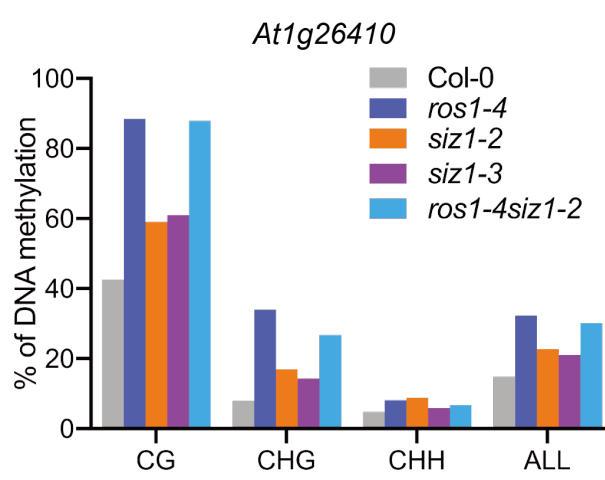
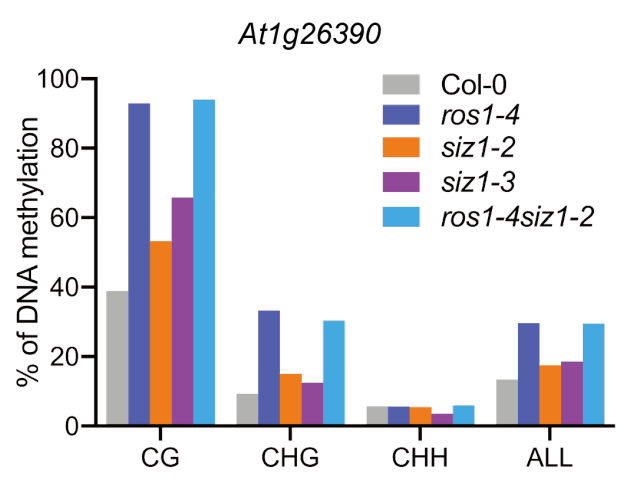
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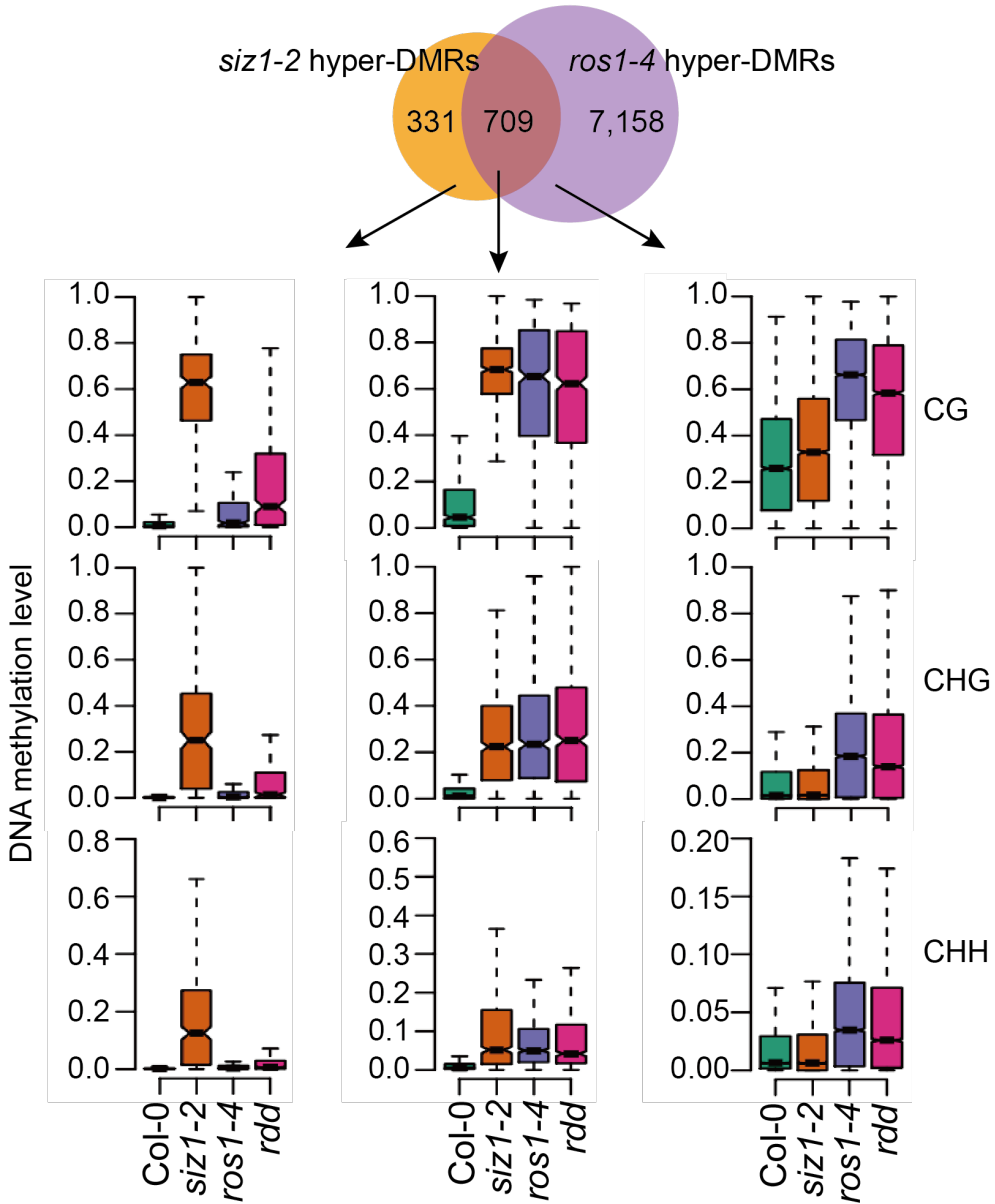
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**A**

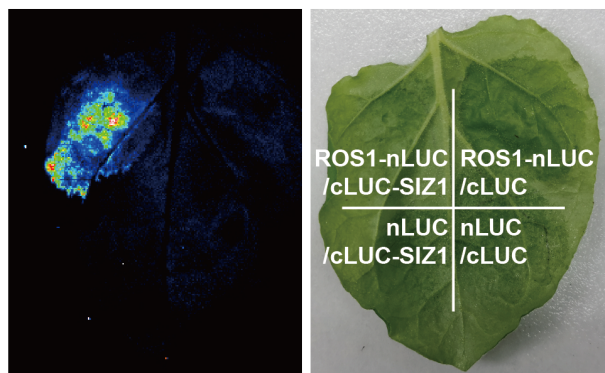
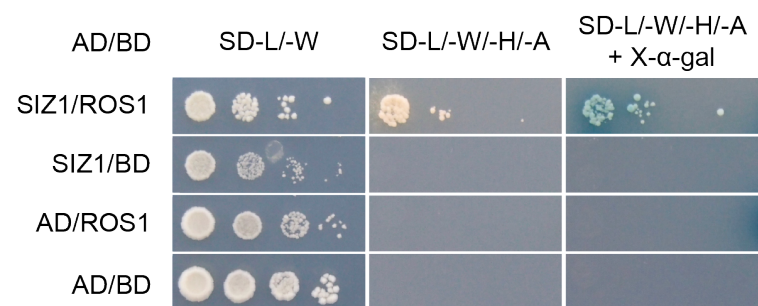
Genotype	Hyper-DMRs	Hypo-DMRs	Overlapped Hyper-DMRs in <i>ros1-4</i>	Overlapped Hyper-DMRs in <i>rdd</i>
<i>siz1-2</i>	1,040	183	709 (68.2%)	768 (73.8%)
<i>ros1-4</i>	7,867	594		5,923 (75.3%)
<i>rdd</i>	11,440	1,530		

**B**

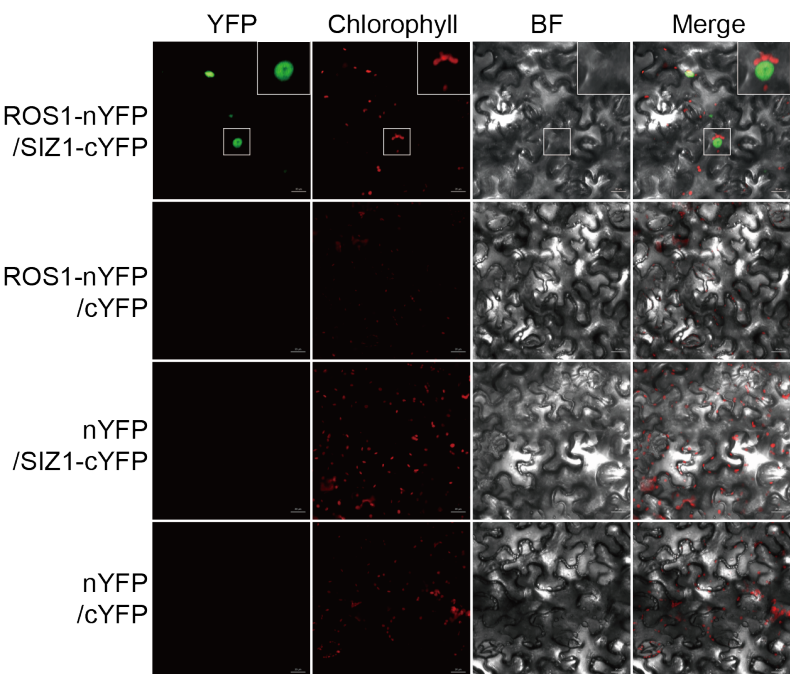




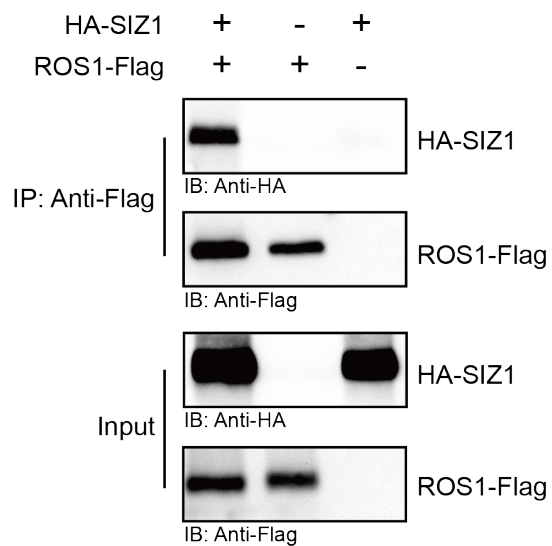
**A**

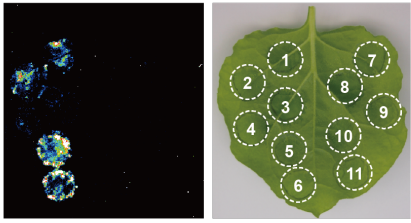
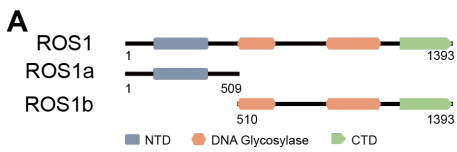


**C**



**D**





- 1/2. ROS1-nLUC/cLUC-SUMO1 8. ROS1a-nLUC/cLUC  
3/4. ROS1a-nLUC/cLUC-SUMO1 9. ROS1b-nLUC/cLUC  
5/6. ROS1b-nLUC/cLUC-SUMO1 10. nLUC/cLUC-SUMO1  
7. ROS1-nLUC/cLUC 11. nLUC/cLUC

