1 **_SlGAD2_ is the target of SlTHM27, positively regulates cold tolerance by**

2 **mediating anthocyanin biosynthesis in tomato**

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19 **Running Head:** _SlGAD2_ affects anthocyanin content and cold tolerance.

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44   **Abstract**

45   Cold stress significantly limits the yield and quality of tomato. Deciphering the key

46   genes related to cold tolerance is important for selecting and breeding superior

47   cold-tolerant varieties. γ-aminobutyric acid (GABA) responds to various types of

48   stress by rapidly accumulating in plant. In this study, glutamic acid decarboxylase

49   (GAD2) was a positive regulator to enhance cold stress tolerance of tomato.

50   Overexpression of *SlGAD2* decreased the extent of cytoplasmic membrane damage

51   and increased the endogenous GABA content, antioxidant enzyme activities, and

52   reactive oxygen species (ROS) scavenging capacity in response to cold stress,

53   whereas *Slgad2* mutant plants showed the opposite trend. In addition, *SlGAD2*

54   induced anthocyanin biosynthesis in response to cold stress by increasing the content

55   of endogenous GABA. Further study revealed that *SlGAD2* expression was negatively

56   regulated by the transcription factor SlTHM27. However, the transcript levels of

57   *SlTHM27* were repressed under cold stress. Antioxidant enzyme activities, *SlGAD2*

58   transcript levels, GABA and anthocyanin contents were significantly increase in

59   *Slthm27* mutant plants. Further, our study demonstrated that SlTHM27 decreases

60   *SlGAD2*-promoted cold resistance in tomato by repressing *SlGAD2* transcription.

61   Overall, our results showed that the SlTHM27-*SlGAD2* model regulates the cold

62   tolerance in tomato by regulating GABA and anthocyanin.

63   **Key words:** *SlGAD2*, *SlTHM27*, GABA, anthocyanin, ROS, cold stress.

64   **INTRODUCTION**

65   Abiotic stresses such as salt, heat, cold, and drought, are among the major factors

66   contributing to the decline in global crop yields and quality [1,2]. Although plants have

67   evolved with the ability to resist environmental stresses, the frequency and intensity

68   of stresses encountered by plants have increased in recent years due to climate change

69   [3,4]. Among these stresses, low temperature is an unavoidable environmental factor that

70   limits agricultural productivity [4]. Below 12°C, high levels of oxidative metabolites

71   accumulate in the plant, affecting the protein and DNA structure, damaging the

72   biofilm and plant tissues, and consequently inhibit the plant growth [5,6]. Various

73   researches have indicated that plants can scavenge ROS generated by enzymatic

74   antioxidant systems (SOD, POD, CAT, APX, etc.) and non-enzymatic antioxidant

75   systems (ASH, GSH, carotenoids, and flavonoids, etc.) under cold stress [6,7].

76   Flavonoids are a class of highly biologically active plant secondary metabolites that

77   have surpassed the performance of some common antioxidants [7]. As active oxygen

78   scavengers, flavonoids reduce free radical damage to plant cells under unfavorable

79   conditions by localizing and neutralizing free radicals [8].

80   Anthocyanins are a class of flavonoids. They not only impart vibrant colors to

81   nutritive tissues such as flowers, leaves, and fruits of the plants, but also act as strong

82   antioxidants for ROS scavenging and against microorganisms in defense reactions [9,10].

83   Anthocyanin biosynthesis includes a series of enzymes such as chalcone isomerase

84   (CHI), chalcone synthase (CHS), flavonoid 3-hydroxylase (F3H), dihydroflavonol

85   4-reductase (DFR), and UDP-glycosidic flavonoid transferase (UFGT) [9,11]. Several

86   transcription factors (TFs) have also been found to regulate the expression of these

87   anthocyanin-synthesizing genes, such as SlANT1 and SlAN2 of the MYB family,

88   SlGL3 and SlTT8 of the bHLH class, HY5 and BBX20 [12-16]. More and more evidence

89   suggested that low temperature induces the expression of anthocyanin synthesizing

90   genes, which in turn boosts the production of anthocyanins, and at the same time, the

91   anthocyanin accumulation can also improve low temperature tolerance of the plants

92   [17,18]. Crifò et al. also discovered that low temperatures promoted anthocyanin

93   accumulation in blood oranges [19]. *MdMYB308L* improved the cold stress tolerance of

94   apple through anthocyanin accumulation [20].

95   Gamma-aminobutyric acid (GABA) acts a key factor in the regulation of plant

96   growth, carbon/nitrogen balance, gene expression, ion homeostasis, and oxidative

97   homeostasis under abiotic stresses [21-23]. Pretreatment with GABA has increased the

98   cold tolerance of tomato and peach fruits [24]. Exogenous GABA significantly

99   up-regulated the expression of WRKY75 and MYB13, and improved the tolerance of

100 *Agrostis stolonifera* L. to drought [25]. Liu et al. discovered that GABA is an effective

101 osmotic agent to reduce reactive oxygen species production in tobacco (*Nictiana*

102 *tabacum* L.) under water stress [26]. In addition, GABA can also alleviate plant damage

103 caused by stresses such as high temperature [22,25], low temperature [27], salt [28], and heavy

104 metals [29] through rapid accumulation. Of course, GABA is also a signaling molecule

105 that activates the phenylalanine pathway and enriches flavonoids, including

106 anthocyanins [30].

107 In plants, glutamic acid decarboxylase (GAD) is the rate-limiting enzyme for

108 GABA synthesis by catalyzing the irreversible synthesis of GABA from glutamic acid

109 (Glu) [28]. The expression of *GAD1* in mulberry leaves is induced by NaCl which

110 promotes the synthesis of GABA, and consequently enhances the salt tolerance of

111 mulberry leaves [31]. The increase transcript levels of *CiGAD1* and *CiGAD2* promoted

112 the accumulation of GABA, and improved the salt stress resistance in mallow [2]. Cold

113 stress significantly increased the GABA content in quinoa [32]. These studies

114 demonstrated that *GAD* is the most sensitive gene for GABA synthesis under abiotic

115 stress. Globally tomato (*Solanum lycopersicum* L.) is a widely grown economic crop

116 with high nutritional value [33]. Since tomato originates from the tropics, the low

117 temperatures negatively affect its growth, yield and quality [33]. Although a lot of

118 studies have been done on how low temperatures alter anthocyanin biosynthesis, the

119 regulation network of GABA content and anthocyanin in cold-stressed tomato

120 remains unclear.

121 Our study revealed that exogenous GABA (55 mM) significantly improved the low

122 temperature tolerance of tomato. In addition, *SlGAD2* was significantly induced by

123 analyzing the transcript levels of GABA synthesis-related genes (*SlGAD1-5*) at low

124 temperature. We also identified SlTHM27, a R2R3 MYB-like TF, responds to cold

125 stress by repressing the expression of *SlGAD2*. Interestingly, endogenous GABA

126 increased anthocyanin accumulation under cold stress. Taken together, we revealed a

127 novel pathway that is SlTHM27-*SlGAD2* to regulate cold stress which might has

128 potential applications in molecular breeding.

129 **Results**

130 **Cold induces GABA accumulation and exogenous GABA enhances cold tolerance**

131 **in tomato**

132 Due to the lack of knowledge about GABA accumulation in tomato seedlings under

133 cold stress, we measured endogenous GABA levels in tomato seedlings at 4°C.

134 GABA levels accumulated significantly with the duration of cold treatment and

135 peaked at 48 h (Fig. S1). To investigate the role of GABA in cold response, the

136 different concentrations of GABA were applied to wild-type (WT) tomato seedlings.

137 Under normal environmental conditions, there was no significant difference in plant

138 height, fresh weight and dry weight of tomato seedlings by exogenous GABA supply

139 compared to the G0 (0 mM GABA) treatment (Fig. 1a, c-f). However, under low

140 temperature treatment, spraying 55 mM GABA resulted in better seedling status

141 compared to the other concentration treatments (Fig. 1a). Further analysis showed that

142 exogenous spraying of 55 mM GABA (G55) significantly increased cold-stressed

143 tomato seedings height, stem thickness, fresh and dry weight (Fig. 1c-f). Ion leakage

144 reflects the extent of stress induced damage to plasma membrane [34]. Compared with

145 the control, low temperature treatment significantly increased the ion leakage and

146 MDA content in tomato seedling. Exogenous spraying of 55 mM GABA resulted in a

147 significant decrease in ion leakage and MDA levels of the seedlings at low

148 temperature (Fig. 1g, h). At the same time, we found that under low temperature stress

149 tomato seedlings accumulated a large amount of H_2O_2 whereas its accumulation in the

150 GABA (55 mM) treated seedlings was significantly reduced (Fig. 1b, i). In conclusion,

151 cold promoted the accumulation of GABA in tomato, while exogenous 55 mM GABA

152 attenuated the cold-induced injury.
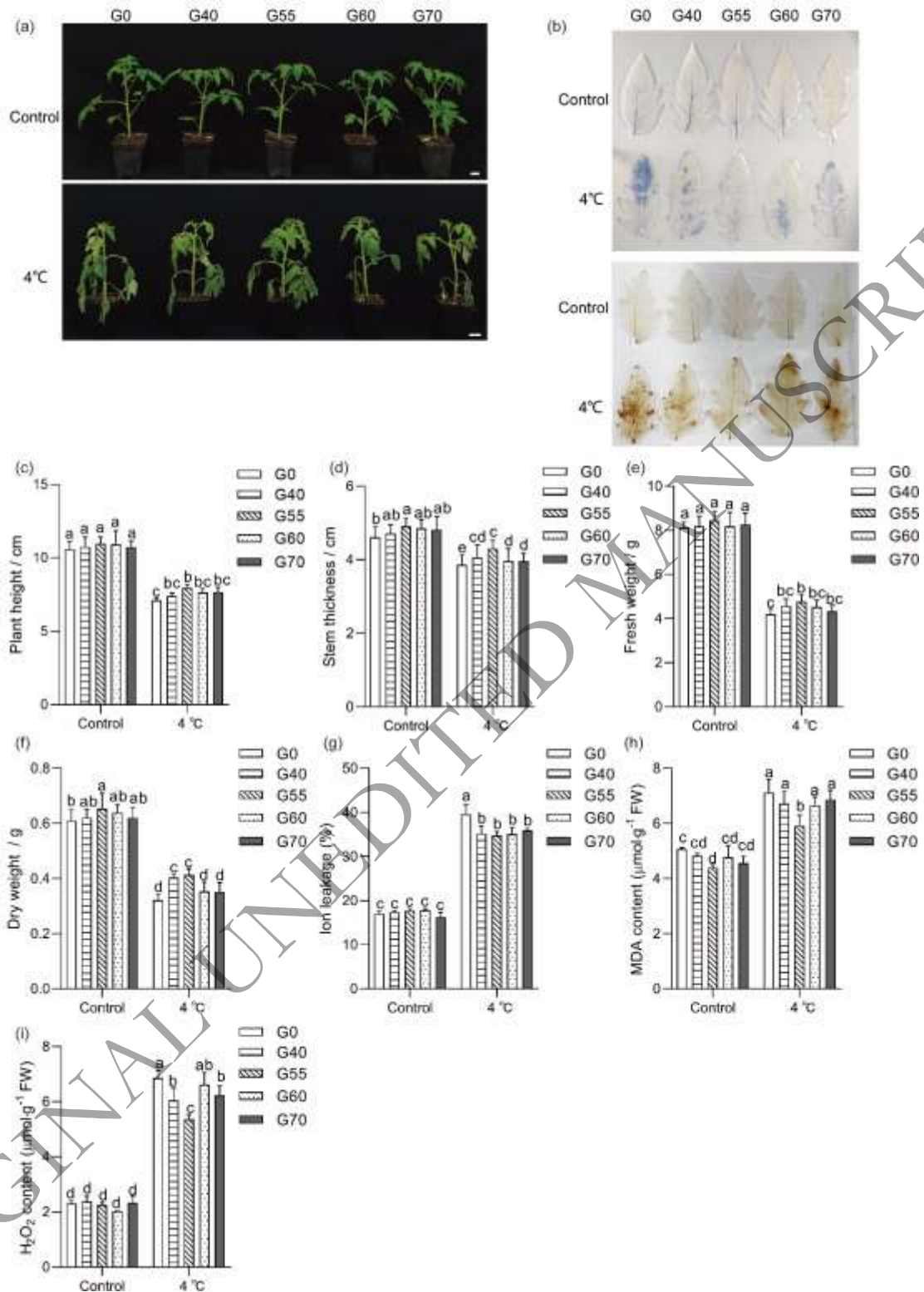
Fig. 1 Exogenous γ-aminobutyric acid (GABA) on the cold tolerance of tomato seedlings.
(a) Phenotypic changes in cold tolerance of tomato seedlings treated with different concentrations
of GABA. Bar, 2.5 cm. G0, G40, G55, G60 and G70 mean exogenous sprays of 0 mM, 40 mM, 55
mM, 60 mM and 70 mM of GABA, respectively. (b) Nitroblue tetrazolium (NBT) and
diaminobenzidine (DAB) staining of leaves with water or GABA-treated plants under control and
cold treatment (4°C) for 4 days. (c-f) Changes in plant height (c), stem thickness (d), fresh (e) and

160 dry weight (f) of water or GABA-treated plants under control and cold treatment (4°C) for 4 days.
161 (g-i) ion leakage (g), MDA (h), and H_2O_2 (i) of water or GABA-treated plants measured before or
162 after cold treatment. Values represent the average of six (c-f) or four (g-i) independent
163 measurements, and error bars represent standard errors. Different letters of the columns indicate
164 significant differences (P <0.05).

165 **_SlGAD2_ is induced by cold stress in tomato**

166 To reveal the genes involved in cold-induced GABA accumulation, we cloned five

167 *GAD* genes with 86.28% sequence alignment identity and high homology in the

168 conserved regions (Fig. S2). Under normal environmental conditions, the expression

169 of five *GAD* homologs was analyzed in different tissues of WT. There were

170 significant differences in the transcript levels of the five *GADs* in different tomato

171 tissue. Among them, *SlGAD1* was heavily induced in leaves, flowers and fruits (Fig.

172 2a); *SlGAD5* was more highly expressed in flowers than in other tissues (Fig. 2e).

173 *SlGAD3* and *SlGAD4* were significantly induced in leaves and flowers (Fig. 2a-d).

174 *SlGAD2* was highly expressed in all tissues (Fig. 2b).

175 Four *GADs* (*SlGAD1*, *SlGAD2*, *SlGAD3*, and *SlGAD4*) with high transcript levels

176 in tomato leaves were explored in response to cold stress. RT-qPCR results showed

177 that all four selected *GADs* were induced under low temperature treatment, but

178 compared with the other three *SlGADs*, only *SlGAD2* was most significantly induced

179 and continuously upregulated (Fig. 2f).
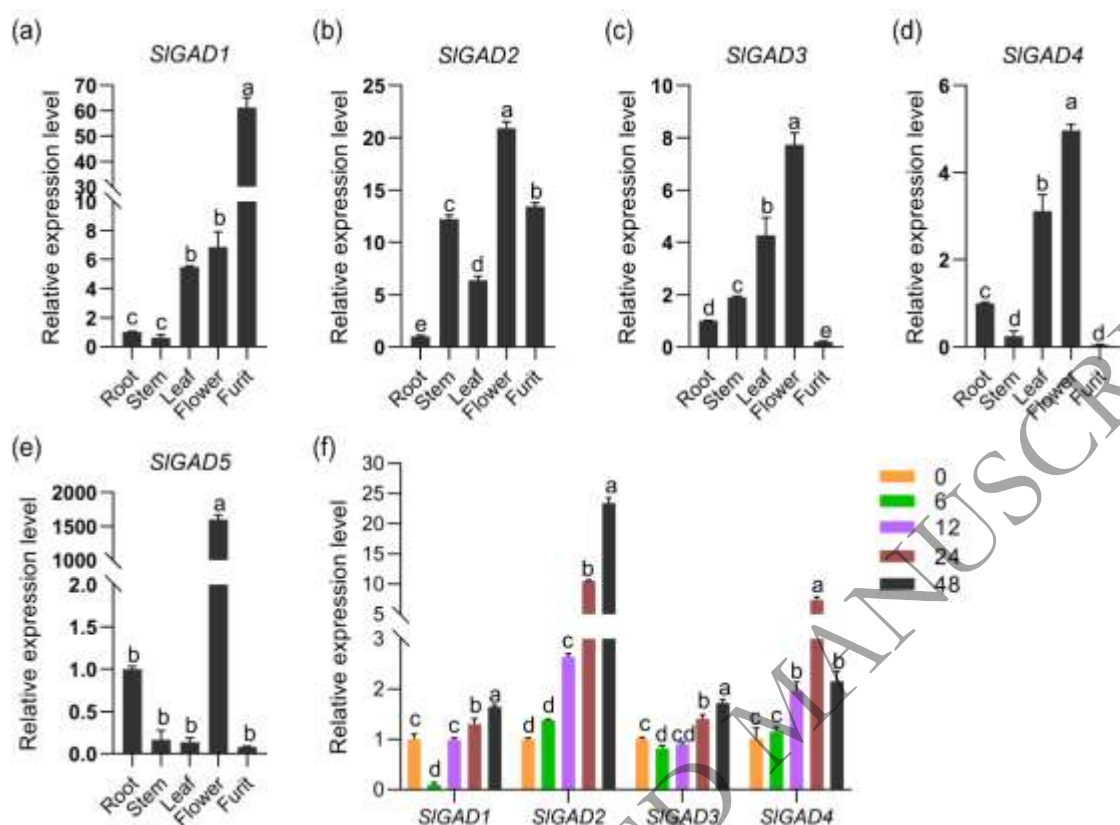
Fig. 2 *SlGAD2* **was significantly induced by cold stress.** (a-e) Changes in transcript levels of the
GABA synthesis-related genes *SlGAD1* (a), *SlGAD2* (b), *SlGAD3* (c), *SlGAD4* (d), and *SlGAD5* (e)
in roots, stems, leaves, flowers, and ripe fruits of tomato. (f) Changes in relative expression of
SlGAD1-5 in leaves of tomato under cold treatment. 0 h in all genes be set to 1. Different letters of
the columns indicate significant differences (P <0.05).

SlGAD2 positively regulates anthocyanin synthesis and antioxidant enzyme activities to enhance tomato cold tolerance

To explore the role of *SlGAD2* in low temperature tolerance, we obtained two
SlGAD2 overexpressing transgenic lines (*SlGAD2* OE#4 and *SlGAD2* OE#5) in the
'Ailsa Craig' tomato background. It was also confirmed that *SlGAD2* expression was
significantly increased in both transgenic lines (Fig. S3). After 4 days of exposure to
low temperature (4°C), the *SlGAD2* OE#4 and *SlGAD2* OE#5 exhibited a cold
tolerance phenotype as compared to WT (Fig. S4a). Meanwhile, *SlGAD2*
overexpression plants had higher GABA level than WT under normal conditions,
whereas low temperature increased GABA accumulation, especially in the *SlGAD2*
overexpression plants (Fig. S4b). Under low temperature treatment, the *SlGAD2*
overexpressed lines had lower ion leakage, MDA content, H_2O_2 and O_2^- accumulation
as compared to WT (Fig. S4c-f). In addition, *SlGAD2*-overexpressing lines also have

199   higher SOD, POD, and CAT activities than WT (Fig. S4g-i), which is consistent with

200   their phenotype of higher cold tolerance.

201   Surprisingly, under low-temperature stress we found pigmentation near the veins

202   in the leaves of *SlGAD2* overexpressed plants (Fig. S5a). Based on quantitative

203   analysis of anthocyanin levels, anthocyanin levels in the leaves of overexpressed

204   *SlGAD2* plants were higher than those of WT under normal conditions and were

205   especially more pronounced under cold stress (Fig. S5b, c). The transcript levels of

206   genes involved in anthocyanin synthesis were also analyzed. The RT-qPCR results

207   showed that low temperature treatment induced the transcription of *SlCHS*, *SlF3H*,

208   *SlDFR* and *SlUFGT*, and it was more so in the *SlGAD2* overexpressed plants (Fig.

209   S5d-g). In summary, overexpression of *SlGAD2* significantly increased antioxidant

210   enzyme activities and anthocyanin level in transgenic plants, leading to improved cold

211   tolerance.

212   To further verify the relationship between GABA levels and anthocyanin

213   accumulation, we examined the endogenous GABA and anthocyanin levels in WT

214   tomato leaves after exogenous application of GABA (55 mM). The results showed

215   exogenous spraying of GABA significantly increased the anthocyanin content

216   compared with the control, and this difference was more pronounced under cold stress

217   (Fig. S6a). In addition, exogenous sprayed GABA significantly increased the

218   anthocyanin content of tomato leaves (Fig. S6b). Thus, the accumulation of

219   endogenous GABA helped to promote the increase of anthocyanins in tomato leaves.

220   To further confirm that the cold-tolerant phenotype of *SlGAD2* OE is caused by

221   enhanced *SlGAD2* function, we constructed *SlGAD2* mutants using CRISPR-Cas9

222   mediated targeting mutagenesis in the "AC" background and selected two mutants

223   without the CRISPR-Cas9 transgene for low temperature treatment (Fig. S7). Under

224   cold stress, *Slgad2* mutant plants exhibited a cold-sensitive phenotype compared to

225   WT (Fig. 3a). Meanwhile, the GABA content of *Slgad2* mutant plants was much

226   lower than that of WT both under normal culture conditions and cold treatment (Fig.

227   3b). Compared with WT, *Slgad2* mutant plants had higher ion leakage level, MDA,

228   and H_2O_2 content and lower SOD, POD, and CAT activities under cold stress (Fig.

229   3c-i).

230   Anthocyanin level of *Slgad2* mutant plants were reduced compared to the WT. This

231   difference was more pronounced under cold stress (Fig. 3j). Similarly, the transcript

232   levels of *SlCHS*, *SlF3H*, *SlDFR* and *SlUFGT* were reduced in *Slgad2* mutant plants,

233   especially more significantly under low temperature conditions (Fig. 3k-n). Analysis

234   of these data further supports that *SlGAD2* plays a positive role in tomato cold

235   tolerance and anthocyanin accumulation.
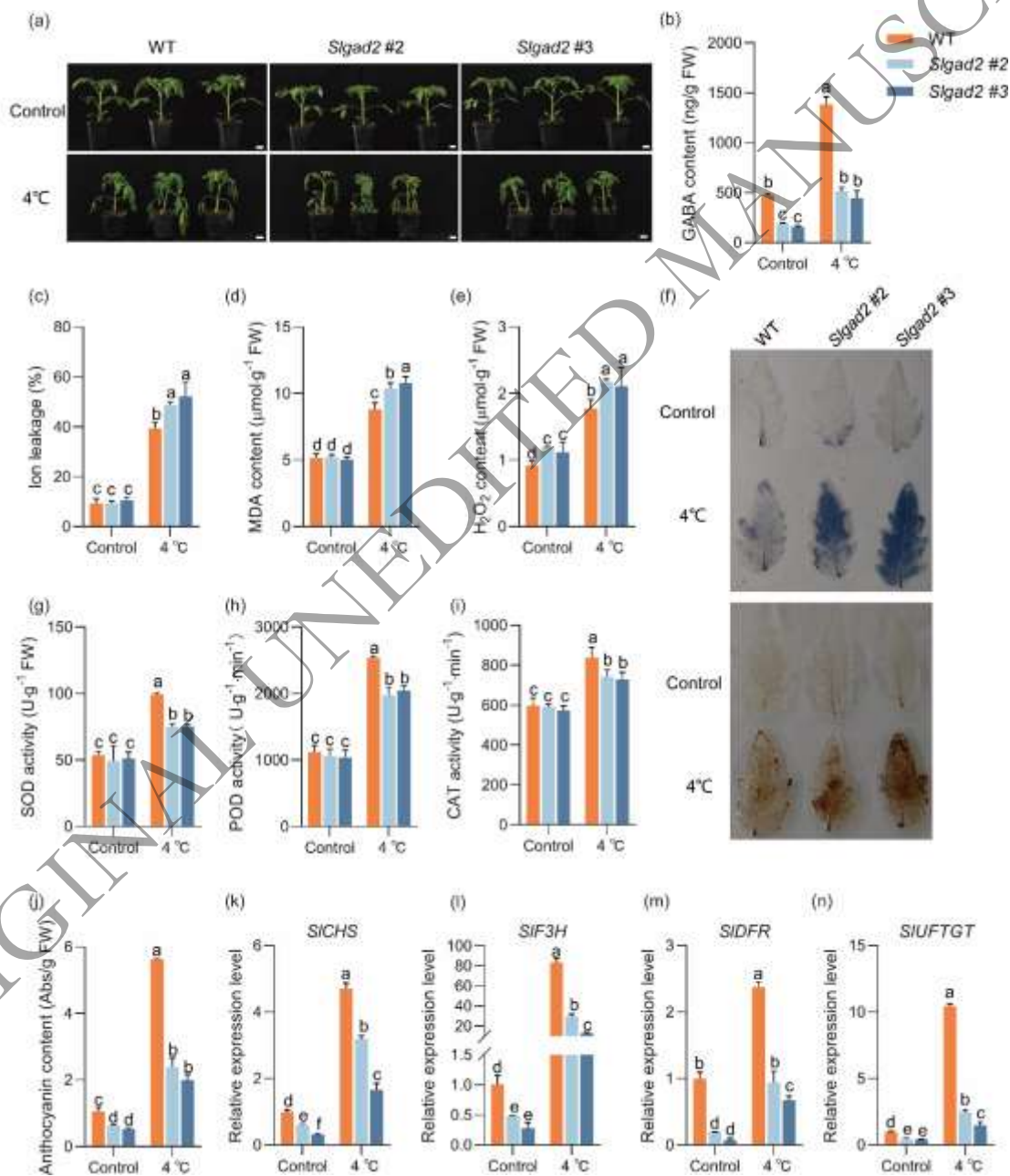
237   **Fig. 3 *Slgad2* mutant plants are more sensitive to cold stress.** (a) Phenotypic changes in WT
238   and *Slgad2* mutant plants treated at low temperature (4°C) for 4 days. Bar, 2.5 cm. (b)
239   Endogenous GABA content tomato leaves shown in (a). (c-e) Electrolyte leakage (c), MDA

240 content (d) and H$_2$O$_2$ content (e) of *Slgad2* mutant plants under control and cold stress (4°C). (f)

241 NBT and DAB staining of leaves from WT and *Slgad2* mutant lines under control and cold stress

242 (4°C). (g–i) SOD activity (g), POD activity (h) and CAT activity (i) in *Slgad2* mutant plants under

243 control and cold stress. Plants were exposed to cold stress for 0 and 4 days, and leaves were

244 collected for the measurements. (j) Anthocyanin content of *Slgad2* mutant plants under control and

245 cold stress (4°C). (k-n) Relative expression levels of *SlCHS*, *SlF3H*, *SlDFR* and *SlUFGT* in *Slgad2*

246 mutant plants under cold treatment for 4 days. *SlACTIN* was used as an internal control. Different

247 letters of the columns indicate significant differences (*P* <0.05).

248 **SlTHM27 is a transcription factor regulating *SlGAD2***

249 To determine the upstream transcription factor of *SlGAD2*, a tomato cDNA library

250 was screened using Y1H with the *SlGAD2* promoter fragment as bait. SlTHM27

251 (Solyc10g055410.1), a protein of the R2R3 MYB family, has been screened and is a

252 homologue of MdMYB16 with 66.67% sequence similarity (Fig. 4a, S8). SlTHM27

253 was only present in the nucleus according to subcellular localization results (Fig. 4b).

254 By searching the PlantTFDB database (http://planttfdb.gao-lab.org/prediction.php),

255 we found that the *SlGAD2* promoter sequence was present with the SlTHM27 putative

256 binding elements TTAGGT and TTTGGT motifs (Fig. 4c). Using an electrophoretic

257 mobility shift assay (EMSA), we found that the SlTHM27-MBP fusion protein was

258 able to bind to the TTAGGT element site but not the TTTGGT element in the *SlGAD2*

259 promoter region (Fig. 4d, S9). Addition of a competing probe resulted in a decrease in

260 binding strength. Mutation of 'TTAGGT' to 'AAAAA' significantly reduced the

261 binding capacity (Fig. 4d). Thus, SlTHM27 binds directly to the promoter of *SlGAD2*.

262 To further characterize the role of SlTHM27 in gene activation, we performed a

263 dual luciferase assay on tobacco leaves. A 1.5 kb *SlGAD2* promoter fragment driving

264 a firefly LUC reporter construct was used to generate an effector construct using

265 SlTHM27 (Fig. 4e). At 25°C, SlTHM27 significantly repressed the expression of

266 *SlGAD2*, but this repression was significantly alleviated after low temperature

267 treatment (Fig. 4e). The qRT-PCR results showed that the transcript levels of

268 *SlTHM27* were all significantly higher than 0h under normal conditions, while the

269 transcript levels of *SlTHM27* were significantly suppressed under low temperature

270 conditions, especially with the lowest expression level of *SlTHM27* at 3h of low

271 temperature treatment (Fig. 4f). Furthermore, we performed sequence comparison

272 with homologous genes in Arabidopsis, apple and tomato and found that SlTHM27

273 has an EAR motif at its C-terminal end (Fig. S10). The EAR motif is a key feature of

274 EAR-type transcriptional inhibitors [35].
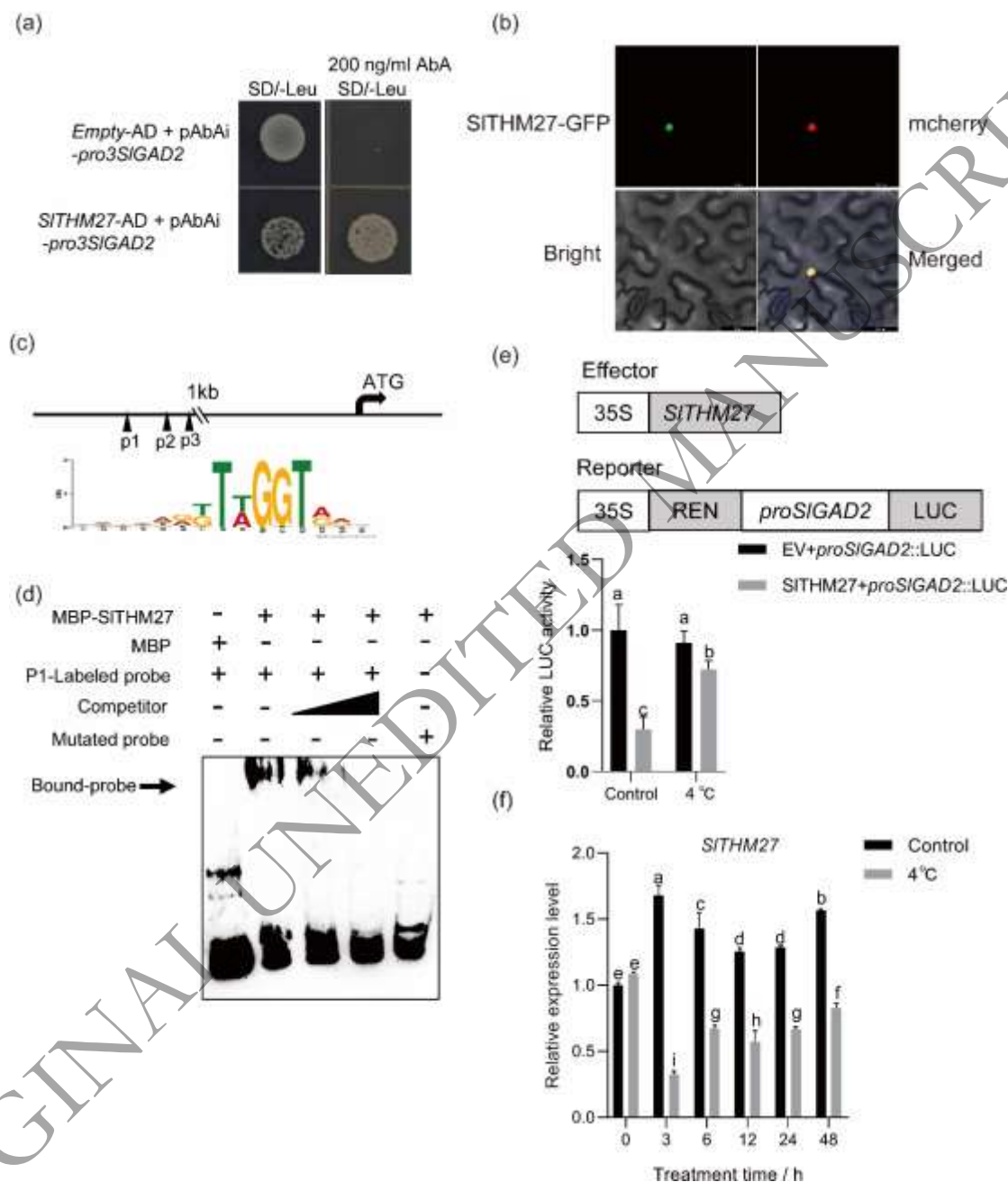
275

276 **Fig. 4 Interaction of SlTHM27 with *SlGAD2* promoter.** (a) An interaction between SlTHM27
277 and promoters of *SlGAD2* by Y1H assays. Empty-AD as a control. (b) Subcellular localization of
278 SlTHM27-GFP and NLS-mCherry in tobacco (Nicotiana benthamiana) leaves. Bar, 50 μM. (c)
279 Schematic representation of the SlTHM27 binding element position on the *SlGAD2* promoter
280 predicted by PlantTFDB. Black triangles represents the predicted positions of the binding
281 elements. p1 represents the 'TTAGGT' binding element, p2 and p3 represent the 'TTTGGT'
282 binding element. (d) SlTHM27-MBP was able to bind to the p1 site of the promoter in *SlGAD2* by
283 EMSA analysis. (e) The inhibition of *SlGAD2* transcription by SlTHM27 was verified by assaying

284 relative LUC/REN activity. The control used was the empty vector pGreen II 62-SK (EV). In cold
285 treatment, infested tobacco was grown under normal conditions for 69 h and then in a growth
286 chamber at 4°C for another 3 h, after which the leaves were collected. (f) *SlTHM27* relative
287 expression changes before and after cold treatment. Different letters of the columns indicate
288 significant differences ($P < 0.05$).

289 **SlTHM27 negatively regulates anthocyanin biosynthesis and antioxidant enzyme**

290 **activities to reduce tomato cold tolerance**

291 Since SlTHM27 has a high sequence similarity (66.67%) to MdMYB16, which was

292 previously reported to negatively regulate anthocyanin synthesis in apple [35], we

293 constructed the *SlTHM27* mutants using CRISPR-Cas9 mediated target mutagenesis

294 (Fig. S11). We evaluated anthocyanin accumulation in tomato leaves of *Slthm27*

295 mutant plants. Anthocyanin content measurements showed that *Slthm27* mutant plants

296 accumulated significantly higher levels of anthocyanins than WT under both normal

297 and low temperature treatments conditions (Fig. 5a). RT-qPCR results showed that

298 *SlCHS*, *SlF3H*, *SlDFR* and *SlUFGT* expression levels were higher in *Slthm27* mutant

299 plants, and this difference was more significant under cold treatments (Fig. 5b-e).

300 Studies have proven a positive correlation between anthocyanins and cold tolerance in

301 plants [17]. We would like to further analyze the role of *SlTHM27* under cold stress in

302 tomato.

303 We found no morphological differences between WT and *Slthm27* mutant plants

304 (*Slthm27* #1 and *Slthm27* #2) under normal conditions. However, the *Slthm27* mutant

305 plants showed a more cold-tolerant phenotype compared to WT under low

306 temperature (Fig. 5f). Meanwhile, *Slthm27* mutant plants showed significantly lower

307 ion leakage and MDA level than control, indicating less damage to membrane lipids

308 compared to the WT under low temperature (Fig. 5h, i). Furthermore, the *Slthm27*

309 mutant lines showed lower accumulation of H_2O_2, O_2^- and higher SOD, POD and CAT

310 activities than WT under cold treatment (Fig. 5j-n). *Slthm27* mutant plants displayed

311 significantly higher levels of *SlGAD2* transcripts and GABA contents under either

312 normal or cold culture conditions (Fig. 5g and Fig. S12). These results suggested that

313 silencing *SlTHM27* promotes GABA accumulation, reduces ROS levels and improves

314 cold tolerance in tomato seedlings. In conclusion, the SlTHM27 negatively regulates

315   anthocyanin synthesis and tolerance to low temperature in tomato.
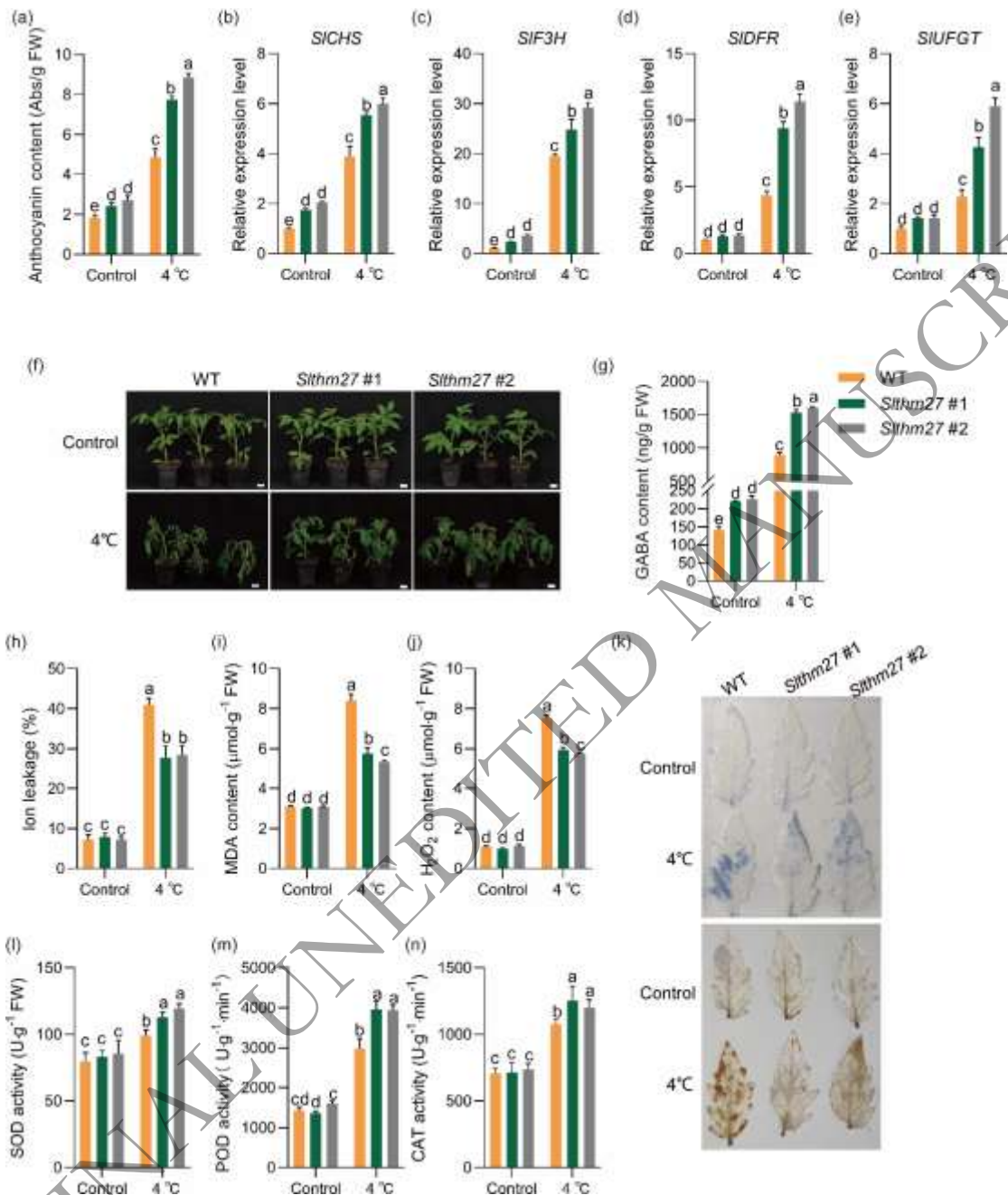
316
317   **Fig. 5 *SlTHM27* negatively regulates cold stress in tomato.** (a) Anthocyanin content of *Slthm27*
318   mutant plants under control and cold stress (4℃). (b-e) Relative expression levels of *SlCHS*,
319   *SlF3H*, *SlDFR* and *SlUFGT* under cold treatment in *Slthm27* mutant plants for 4 days. (f)
320   Phenotypic changes in WT and *Slthm27* mutant plants treated at low temperature (4℃) for 4 days.
321   Bar, 2.5 cm. (g) Endogenous GABA content tomato leaves shown in (f). (h-j) Electrolyte leakage
322   (h), MDA content (i) and H_2O_2 content (j) of *Slthm27* mutant plants under control and cold stress
323   (4℃). Plants were exposed to cold stress for 4 days, and leaves were collected for an ion leakage
324   assay, MDA and H_2O_2 content measurement. (k) NBT and DAB staining of leaves from WT and
325   *SlTHM27*- silenced lines under control and cold stress (4℃). (l–n) SOD activity (l), POD activity
326   (m) and CAT activity (n) in *Slthm27* mutant plants under control and cold stress. Plants were
327   exposed to cold stress for 0 and 4 days, and leaves were collected for the measurements. Different

328   letters of the columns indicate significant differences ($P < 0.05$).

329 SlTHM27 decreases *SlGAD2*-promoted cold tolerance in tomato by repressing

330 *SlGAD2* transcription

331   To further verify that SlTHM27 regulates tomato cold tolerance by regulating

332   *SlGAD2*, we silenced *SlGAD2* in *Slthm27* mutant plants. The results indicated that

333   under cold stress, there was a significant increase in the transcript level of *SlGAD2* in

334   the *Slthm27* mutant compared to the WT with pTRV. In the background of the

335   *Slthm27* mutant, where *SlGAD2* was silenced, the expression of *SlGAD2* was

336   significantly suppressed, although it remained higher than the expression observed in

337   the WT background with pTRV-*SlGAD2* (Fig. S13). We found that compared with

338   pTRV in the WT background, the cold tolerance of pTRV in the *Slthm27* mutant

339   background was significantly enhanced, with reduced ion leakage, MDA and H_2O_2

340   contents, and significantly increased antioxidant enzyme activities (SOD, POD and

341   CAT), along with significantly higher GABA and anthocyanin contents (Fig. 6a-j).

342   However, in the *Slthm27* mutant background, knockdown of *SlGAD2* partially

343   impaired cold tolerance in *Slthm27* mutant plants due to increased ion leakage level,

344   MDA and H_2O_2 levels, and reduced antioxidant enzyme activity (Fig. 6a-i).

345   Furthermore, under low temperature treatment, the GABA content of pTRV-*SlGAD2*

346   in the *Slthm27* mutant background was more similar to that of pTRV in the WT

347   background (Fig. 6b). It suggests that SlTHM27 regulates *SlGAD2* to affect GABA

348   synthesis. Taken together, our results suggest that SlTHM27 negatively regulates cold

349   tolerance in tomato by inhibiting *SlGAD2*-promoted GABA accumulation and

350   anthocyanin biosynthesis.
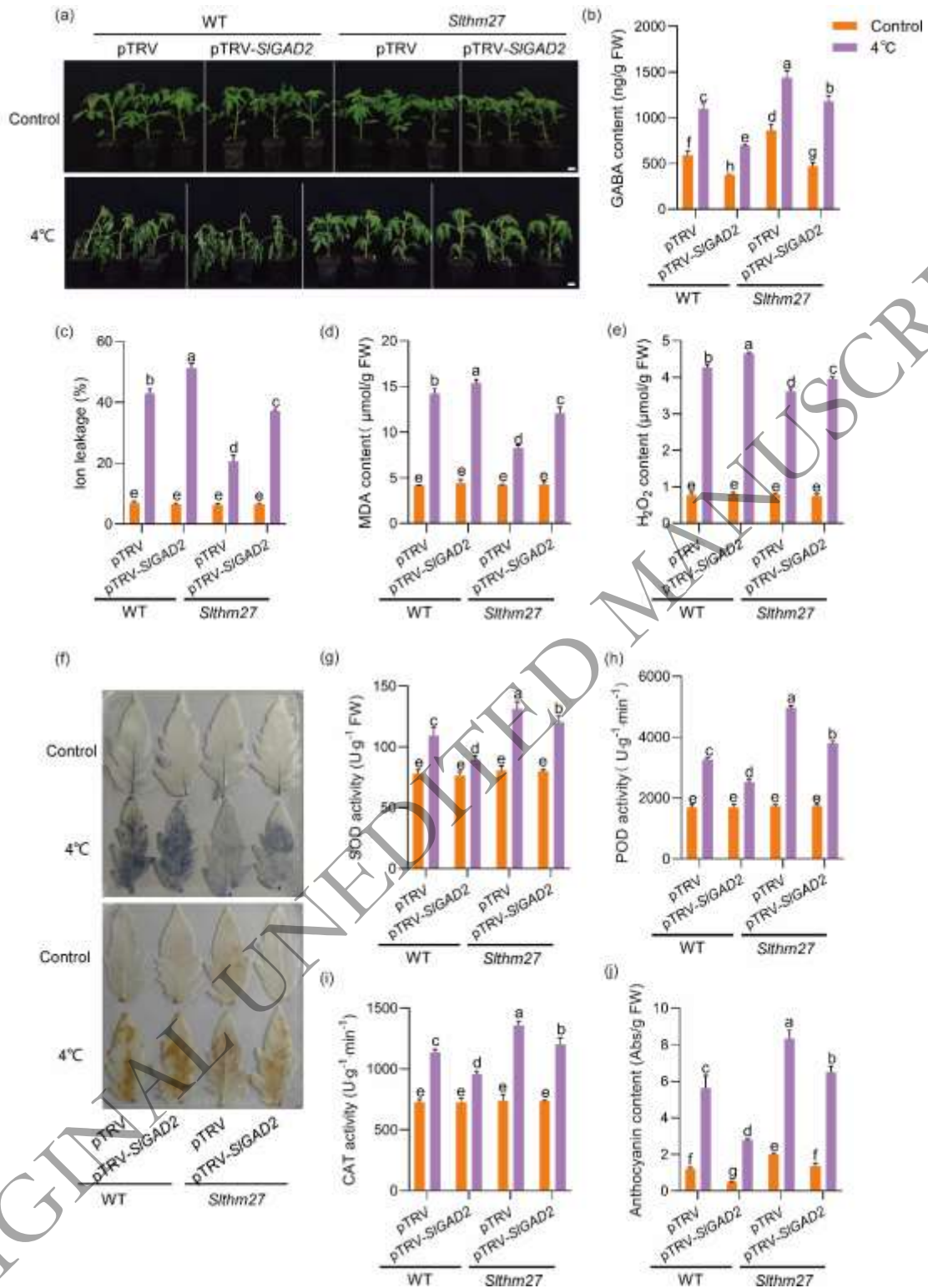
Fig. 6 SlTHM27 negatively regulates cold tolerance in tomato by repressing *SlGAD2* transcription and GABA accumulation. (a) Phenotypic changes of silenced (pTRV-*SlGAD2*) or non-silenced *SlGAD2* (pTRV) in WT and *Slthm27* mutant plants before and after treatment at low temperature (4°C) for 4 days. Bar, 2.5 cm. (b) Endogenous GABA content tomato leaves shown in (a). (c-e) Electrolyte leakage (c), MDA content (d), and H_2O_2 content (e) of silenced (pTRV-*SlGAD2*) or non-silenced *SlGAD2* (pTRV) in WT and *Slthm27* mutant plants under control and cold stress (4°C). Plants were exposed to cold stress for 4 days, and leaves were collected for

359　an ion leakage assay, MDA and H_2O_2 content measurement. (f) NBT and DAB staining of leaves
360　of silenced (pTRV -*SlGAD2*) or non-silenced *SlGAD2* (pTRV) in WT and *Slthm27* mutant plants
361　under control and cold stress (4℃). (g–i) SOD activity (g), POD activity (h), and CAT activity (i)
362　of silenced (pTRV -*SlGAD2*) or non-silenced *SlGAD2* (pTRV) in WT and *Slthm27* mutant plants
363　under control and cold stress. Plants were exposed to cold stress for 0 and 4 d and leaves were
364　collected for the measurements. (j) Anthocyanin content of silenced (pTRV-*SlGAD2*) or
365　non-silenced *SlGAD2* (pTRV) in WT and *Slthm27* mutant plants under control and cold stress
366　(4℃). Different letters of the columns indicate significant differences (P <0.05).

367　**Discussion**

368　**Exogenous spraying of 55 mM GABA effectively improved tomato cold tolerance**

369　Tomato, originally from the tropics, is highly sensitive to low-temperature stress [33].

370　Therefore, elucidating the molecular mechanism of tomato cold sensitivity is

371　important for crop breeding and improvement. GABA is not only a metabolic

372　substance but also a signaling molecule that plays a critical role in mitigating cold

373　injury in various species through accumulation [2,36,37]. In this study, tomato seedlings

374　showed typical symptoms of cold injury under cold stress, including slow growth and

375　reduction in fresh and dry weight, which were effectively alleviated by exogenous

376　spraying of 55 mM GABA (Fig. 1). Moreover, we found that exogenous GABA

377　maintained the integrity of cellular structure by improving the activity of antioxidant

378　enzymes to scavenge ROS (Fig. 1). It was further demonstrated that exogenous

379　GABA could regulate the activation capacity of plant antioxidant defense system

380　under cold stress [37, 38]. Therefore, exogenous spraying of GABA is an effective way to

381　improve the cold tolerance of tomato seedlings during tomato cultivation in facilities.

382　*SlGAD2* **is a positive regulator to improve the cold tolerance of tomato**

383　GAD is a key enzyme in GABA production [28]. Our results showed that the tissue

384　expression of the five *GAD* genes varied greatly, with all four *GAD* genes except

385　*SlGAD5* being transcribed at higher levels in leaves (Fig. 2a-e). *SlGAD1* and *SlGAD2*

386　being more highly expressed in ripe tomato fruits (Fig. 2a, b). Some studies have

387　shown that the expression of *SlGAD1-3* was essential for the synthesis of GABA in

388　tomato fruits [39]. The only difference was that the present study found lower expression

389　of *SlGAD3* in ripe fruits, possibly due to the fact that the mRNA level of *SlGAD3* is

390　highest early in fruit development and decreases with fruit ripening [40]. In addition, this

391 study investigated the transcript levels of *SlGADs* in tomato leaves under cold stress

392 for the first time (Fig. 2). Under cold stress, the transcript levels of *SlGAD1* and

393 *SlGAD3* decreased and then increased, whereas the transcript level of *SlGAD4*

394 showed a concurrent trend of increase and then decrease; only the relative expression

395 of *SlGAD2* increased significantly with increasing stress time (Fig. 2f). It is

396 noteworthy that the relative expression of *SlGAD2* was basically consistent with the

397 dynamic changes of GABA content under cold stress. This implied that *SlGAD2* plays

398 a crucial role in change of the GABA content of tomato seedlings under cold stress.

399 **SlGAD2 positively regulates the cold tolerance in tomatoes by scavenging ROS**

400 **and increasing anthocyanin content**

401 We further investigated the mechanism of action of *SlGAD2*, given that *SlGAD2* was

402 the most sensitive to cold stress. We found that overexpression of *SlGAD2* increased

403 the cold tolerance of tomato seedlings by increasing GABA content and antioxidant

404 capacity (Fig. S4). On the contrary, *Slgad2* mutants showed the oppsite trend (Fig. 3).

405 This was consistent with the conclusion that exogenous GABA improves cold

406 tolerance by increasing antioxidant capacity in banana fruits [38]. However, it is not

407 clear whether GABA improves cold resistance in tomato through other pathways. In

408 this study, we found that anthocyanin content was significantly accumulated in

409 *SlGAD2*-overexpressing plants (Fig. S5). Anthocyanins are a major class of

410 flavonoids whose synthesis and accumulation are induced by low temperature [17,18].

411 The anthocyanin content and the expression of anthocyanin-related genes were

412 significantly increased in *SlGAD2* overexpressing plants under low-temperature stress,

413 whereas the anthocyanin content was significantly suppressed in *Slgad2* mutant plants

414 (Fig. 3j-n). Anthocyanins can effectively scavenge excessive ROS to maintain normal

415 cellular redox homeostasis under abiotic stress [10]. Therefore, anthocyanins

416 accumulated in the overexpressed *SlGAD2* transgenic tomato attenuated low

417 temperature induced oxidative damage. At the same time, we found that spraying 55

418 mM GABA increased the content of endogenous GABA, which also promoted the

419 anthocyanin accumulation and improved cold tolerance in tomato (Fig. S6). These

420 results further demonstrated that GABA accumulation could efficiently scavenge ROS

421 through both enzymatic and non-enzymatic antioxidant systems under cold stress, and

422 *SlGAD2* played an essential role in this process. In addition, it will be interesting to

423 study how GABA triggers the expression of anthocyanin synthesis genes.

424 **SlTHM27 negatively regulated cold by inhibiting *SlGAD2* transcription**

425 Most of the MYB class TFs can respond positively to plant tolerance to abiotic

426 stress [41], only a few MYB class TFs were negative regulators of abiotic stress

427 response [42,43]. For example, VcMYB4a, which has an EAR repressor domain of

428 structure, was down-regulated by low-temperature treatment, and blueberry healing

429 tissues exhibited a cold-sensitive phenotype after *VcMYB4a* overexpression [44].

430 Similarly, we found that the SlTHM27 protein sequence contained a C-terminal EAR

431 repressor motif (Fig. S10), and its mutant plants exhibited enhanced cold tolerance

432 (Fig. 5). In addition, MYB class transcriptional activators and repressors have been

433 widely explored in the regulation of anthocyanin biosynthesis [35,45]. AtMYB4, which

434 contains an EAR motif, represses C4H expression [46]. MdMYB16 inhibits anthocyanin

435 synthesis in apple healing tissues by repressing *MdANS* and *MdUFGT* expression [35].

436 In this study, we found that SlTHM27 is an MdMYB16 homolog with 66.67% protein

437 sequence similarity. SlTHM27 was found to repress *SlGAD2* transcription by

438 qRT-PCR and Dual-LUC assays (Fig. 4e, S12). Furthermore, our results convincingly

439 demonstrated that *SlGAD2* acts downstream of SlTHM27 (Fig. 4, 6). Thus, the

440 enhanced cold tolerance of *Slthm27* mutant plants depends on the increased transcript

441 level of *SlGAD2*, which in turn promotes GABA accumulation and improves

442 anthocyanin content and ROS scavenging (Fig. 5, 6).

443 Taken together, our work has revealed for the first time the mechanism of the

444 SlTHM27-*SlGAD2* regulatory module responds to cold stress by regulating GABA

445 levels (Fig. 7). Cold stress inhibited the mRNA level of the negative regulator

446 *SlTHM27* to weaken the transcriptional repression of *SlGAD2* and induced the

447 synthesis of GABA to improve tomato resistance through enzymatic and

448 non-enzymatic antioxidant systems. Our study provides valuable insights for

449 improving cold tolerance in tomato.
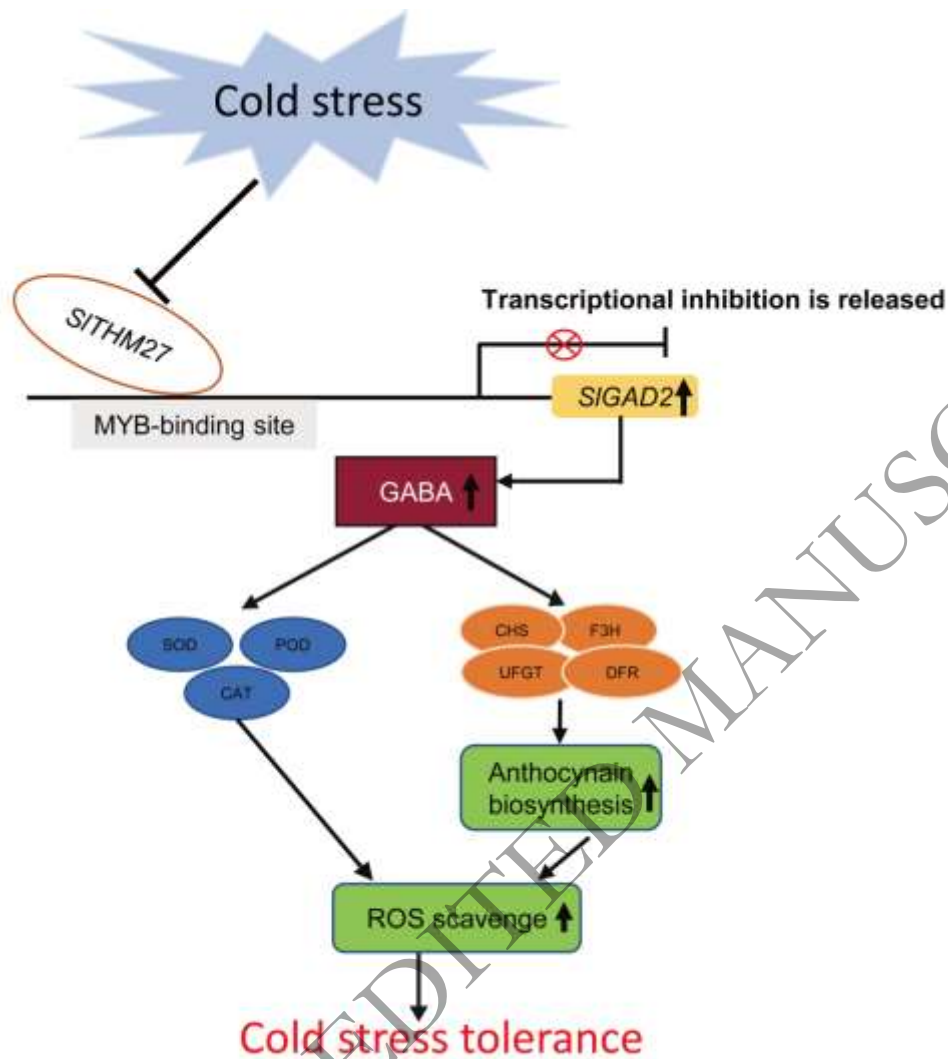
450

451 **Fig. 7 A working model for SlTHM27-*SlGAD2* in response to cold stress.** Under
452 low-temperature stress, the transcript level of *SlTHM27* was repressed thereby increasing the
453 expression level of *SlGAD2*. *SlGAD2* promoted the accumulation of GABA, which increased
454 antioxidant enzyme activities and anthocyanin levels. This helped to scavenge excess reactive
455 oxygen species (ROS) and improved cold tolerance in tomato.

456 **MATERIALS AND METHODS**

457 **Vector construction and genetic transformation**

458 The CDS amplicon of *SlGAD2* gene (ID: Solyc11g011920.1) was inserted into
459 pHellsgate2 vector, and the fusion-expressing vector was introduced into the tomato
460 'Ailsa Craig' (WT) to achieve genetic transformation. The *SlGAD2* overexpression
461 plants were produced and identified by PCR. The primers used in this research are
462 listed in Table S1.

463 To edit the *SlTHM27 and SlGAD2* genes using the CRISPR/Cas9 system. Targets

464 were designed and selected using Cas-Designer

465 (http://www.rgenome.net/cas-designer/). The guide RNA sequence was constructed

466 and inserted into pBSE402 [47]. Tomato transformation was performed according to

467 previously described methods. Positively transformed plants were identified by

468 extracting genomic DNA from stable transgenic lines, cloning potential editing

469 fragments of *SlTHM27 and SlGAD2*, respectively, and sequencing them.

470   For gene silencing, primers listed in Table S2 were used to amplify cDNA

471 fragment of *SlGAD2*, and the PCR amplification product were then transformed into

472 the TRV2 vector. The fusion expression vector was transfected into wild-type tomato

473 cotyledons to generate *SlGAD2* gene-silencing plants. For details of the method,

474 please refer to our group's previous study [48].

475 Plant materials and treatment

476   We germinated both WT and transgenic tomato seeds at 28°C, after which they

477 were planted separately in cavity trays and cultured in a light incubator under culture

478 conditions: 25°C/16°C (day/night). Seedlings were transplanted to nurseries with four

479 fully expanded true leaves, and continued to be grown until the fifth true leaf was

480 fully expanded under environmental conditions as described previously. For the cold

481 treatment, tomato seedlings were incubated in a cold chamber at 4°C for 4 days.

482   In the exogenous GABA treatment assays, leaves were uniformly sprayed with

483 distilled water (0 mM GABA) or GABA solutions of appropriate concentrations, 10

484 mL plant^{-1}. Leaves were treated for 12 h at 25°C and then subjected to a low

485 temperature treatment (4°C). The GABA concentrations applied included C0 (0 mM

486 GABA), C40 (40 mM GABA), C55 (55 mM GABA), C60 (60 mM GABA), and C70

487 (70 mM GABA).

488 RT-qPCR analysis

489   Total RNA was extracted with tomato leaves, and reverse transcription did with

490 PrimeScriptTM RT Kit (Takara Bio, Shiga, Japan). RT-qPCR analysis was performed

491 with ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China). *SlACTIN* was

492 employed as parameter.

493 Bioinformatics analysis

494   DNAMAN was used for multiple amino acid comparisons of protein sequences,

495   and the SMART program was used to analyze conserved protein structural domains [49].

496   The online tool PlantTFDB (http://planttfdb.gao-lab.org/prediction.php) was used to

497   analyze the TF binding sites in the *SlGAD2* promoter.

SlTHM27-GFP subcellular localization

499   The CDS sequences of *SlTHM27* was inserted into pAC402-GFP. The fusion

500   proteins were transferred to one-month-old tobacco leaves. GFP signal was detected

501   by laser scanning confocal microscopy (TCS-SP8 SR; Leica, Wetzlar, Germany).

502   NLS-mCherry was used to as the nucleus marker.

Yeast one-hybrid (Y1H) assay

504   The *SlGAD2* promoter was introduced into the pAbAi vector and then digested

505   with BbsI (NEB, IpswichI, MA, USA) as a bait. The CDS sequences of *SlTHM27* was

506   cloned into pGADT7 as a prey vector. Y1H experiments were performed according to

507   the Matchmaker Gold Y1H manufacturer's instructions. Table S1 lists the primers

508   used for amplification.

Dual-luciferase reporter assay system

510   For the LUC assay, the fusion reporter gene (*proSlGAD2*) and effector (*SlTHM27*)

511   plasmids were inserted into *Agrobacterium tumefaciens* GV3101, respectively.

512   One-month-old tobacco leaves were transiently transformed as described previously [50].

513   A dual luciferase reporter assay system (Promega) was used to detect luciferase

514   activity. In low temperature treatment, tobacco plants were subjected to treament at

515   4°C for 3 h and then proteins were extracted. Ten independent biological samples

516   were used.

EMSA

518   The truncated *SlTHM27* was cloned into pMAL-c5X. The resulting plasmid was

519   converted into *E. coli* strain BL21 (DE3) and amplified for 8 h at 28°C.

520   MBP-SlTHM27 fusion protein was purified using straight-chain starch resin (NEB,

521   E8201S, USA). The EMSA assays were carried out with the Light Shift

522   Chemiluminescent EMSA Kit (ThermoFisher Scientific). Table S1 lists the primers

523   used for amplification.

Determination of GABA content

GABA was extracted using 0.1 g of tomato leaves, and GABA content was measured by LC-MS [51]. GABA content was analyzed using three independent experiments each having three replicates.

Determination of total anthocyanin content

Total anthocyanins were extracted from tomato leaves using a solution of methanol and HCl (0.1%, v/v) at 4°C overnight. Absorbance was detected at 530, 657 nm using a UV-Vis spectrophotometer (Shimadzu UV-1780) [10].

H_2O_2, Ion leakage, enzymes activities and MDA content measurements

H_2O_2 were determined as previously described by Xie et al. [50]. Ion leakage was measured according to the method of Jiang et al. [52]. SOD, POD, CAT activities and MDA levels were calculated as previously described [29].

DAB and NBT staining of the tomato leaves

Tomato leaves were analyzed for H_2O_2 by placing them in a 1 mg mL^{-1} solution of 3,3'-diaminobenzidine (DAB) (pH 3.8) in the light for 8 h. For O_2^- analysis, the tomato leaves were immersed in a 0.5 mg mL^{-1} solution of nitroblue tetrazolium (NBT) in the dark for 8 h.

Determination of growth

The height of tomato seedlings were measured in cm using a meterstick and stem thickness in mm using a Vernier caliper. Fresh and dry weights of tomato seedlings were measured using a balance. Accuracy to one thousandth of a millimeter.

Statistical analysis

Data presented as mean ± SD. Statistical analysis was performed with SPSS 23.0. One-way ANOVA was used to consider differences significant at *P<0.05* or *P<0.01* (Tukey's test).

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559 **Author contributions**

560 X.H., T.L., J.W. and Y.Z. conceived and designed the experiments. J.W. and X.H.

561 wrote the paper. J.W. and Y.Z. performed the experiments. J.W., A.K., Z.K., Y.M., J.Z.

562 and H.D. provided advice related to the research. All authors read and approved the

563 manuscript for submission.

564 **Data availability statement**

565 All relevant data for this study are provided in this article and its supplements.

566 **Conflict of interests**

567 The authors declare no competing interests.

568 **Supplementary information**

569 Supporting Tables:

570 Table S1 List of primers used in this study.

571 Table S2 Primers used for quantitative real-time PCR.

572 Supporting Figures:

573 Fig. S1 Endogenous GABA content tomato leaves at designed time points of cold

574 treatment.

575 Fig. S2 Multiple amino acid sequence alignment of SlGAD1-5.

576 Fig. S3 Relative expression levels of *SlGAD2* in transgenic tomatoes of

577 overexpression *SlGAD2* (*SlGAD2 OE*#4 and *SlGAD2 OE*#5).

578 Fig. S4 *SlGAD2* OE plants are more tolerant to cold stress.

579 Fig. S5 *SlGAD2* induces anthocyanin synthesis in tomato seedlings.

580 Fig. S6 GABA induces anthocyanin accumulation under low-temperature stress.

581 Fig. S7 CRISPR - Cas9 mediated target mutations in *SlGAD2*.

582 Fig. S8 Multiple amino acid sequence alignment of SlTHM27 and MdMYB16.

583   Fig. S9 SlTHM27-MBP was not able to bind the 'TTTGGT' element on the

584   *SlGAD2* promoter by EMSA analysis.

585   Fig. S10 Multi-alignment of the amino acid sequences of MYB type transcription

586   repressors.

587   Fig. S11 CRISPR - Cas9 mediated target mutations in *SlTHM27*.

588   Fig. S12 Relative expression level of *SlGAD2* in *Slthm27* mutant plants under

589   control and cold stress for 4 days.

590   Fig. S13 Relative expression level of *SlGAD2* under silencing (pTRV-*SlGAD2*) or

591   non-silencing *SlGAD2* (pTRV) conditions in WT and *Slthm27* mutant plants.

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