

1 **Constitutive Steroidal Glycoalkaloid Biosynthesis in Tomato is Regulated by the Clade IIIe**
2 **Basic Helix-Loop-Helix Transcription Factors MYC1 and MYC2^[W]**

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23 MYC2 control the constitutive biosynthesis of tomato steroidal glycoalkaloids and might do so
24 independently of jasmonate signaling.

25 Footnotes:

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33 **ABSTRACT**

34 **Specialized metabolites are produced by plants to fend off biotic enemies. Across the plant kingdom,**
35 **the biosynthesis of these defense compounds is promoted by jasmonate signaling in which clade IIIe**
36 **basic helix-loop-helix (bHLH) transcription factors take on a central role. Tomato (*Solanum***
37 ***lycopersicum*) produces cholesterol-derived steroidal glycoalkaloids (SGAs) that act as**
38 **phytoanticipins against a broad variety of herbivores and pathogens. The biosynthesis of SGAs**
39 **from cholesterol occurs constitutively in tomato plants and can be further stimulated by jasmonates.**
40 **Here, we demonstrate that the two tomato clade IIIe bHLH transcription factors, MYC1 and**
41 **MYC2, redundantly and specifically control the constitutive biosynthesis of SGAs. Double *myc1***
42 ***myc2* loss-of-function tomato hairy roots displayed suppressed constitutive expression of cholesterol**
43 **and SGA biosynthesis genes, and consequently severely reduced levels of the main tomato SGAs α -**
44 **tomatine and dehydrotomatine. In contrast, basal expression of genes involved in canonical**
45 **jasmonate signaling or in the biosynthesis of highly jasmonate-inducible phenylpropanoid-**
46 **polyamine conjugates was not affected. Furthermore, CRISPR-Cas9(VQR)-mediated genome**
47 **editing of a specific *cis*-regulatory element, targeted by MYC1/2, in the promoter of a cholesterol**
48 **biosynthesis gene led to decreased constitutive expression of this gene, but did not affect its**
49 **jasmonate inducibility. Our results demonstrate that clade IIIe bHLH transcriptional regulators**
50 **might have evolved to regulate the biosynthesis of specific constitutively accumulating specialized**
51 **metabolites independent of jasmonate signaling.**

52 INTRODUCTION

53 Plants produce species-specific specialized metabolites to defend themselves against natural
54 enemies, such as herbivores and pathogens. Several *Solanaceae* species, including tomato
55 (*Solanum lycopersicum*), potato (*S. tuberosum*) and eggplant (*S. melongena*), synthesize steroidal
56 glycoalkaloids (SGAs). These nitrogen-containing compounds with a terpenoid skeleton
57 accumulate constitutively in many plant organs, thereby forming a chemical barrier that helps the
58 plant to protect itself against a broad range of biotic agents (Friedman, 2002). The main tomato
59 SGAs, α -tomatine and dehydrotomatine, are present in nearly all plant organs, including leaves,
60 roots, flowers and immature green fruits (Friedman and Levin, 1998; Kozukue et al., 2004). As
61 the fruits mature and ripen, *de novo* SGA synthesis terminates and α -tomatine and
62 dehydrotomatine are converted into esculeosides and dehydroesculeosides, respectively, the
63 predominant SGAs in ripe fruits (Yamanaka et al., 2009; Cárdenas et al., 2019; Nakayasu et al.,
64 2020).

65 Cholesterol, which in plants is derived from cycloartenol, is the biosynthetic precursor for
66 SGAs. Cycloartenol is produced via the cytosolic mevalonate pathway and forms the branch
67 point between cholesterol and phytosterol biosynthesis. Gene duplication and divergence from
68 phytosterol biosynthetic genes accounts for half of the cholesterologenesis genes (Sawai et al.,
69 2014; Sonawane et al., 2016). The other cholesterol biosynthesis genes are shared between the
70 cholesterol and phytosterol pathway (Sonawane et al., 2016). A set of *GLYCOALKALOID*
71 *METABOLISM (GAME)* genes that are partially organized in metabolic gene clusters, are
72 responsible for the biosynthesis of SGAs from cholesterol (Itkin et al., 2011; Itkin et al., 2013;
73 Sonawane et al., 2018). In a first series of reactions catalyzed by a subset of the GAME proteins,
74 cholesterol is converted into SGA aglycones via multiple biosynthetic steps, including the
75 transamination of the sterol backbone (Itkin et al., 2013; Sonawane et al., 2018). Subsequently,
76 these steroidal alkaloids are glycosylated by uridine diphosphate (UDP) glycosyltransferases to
77 form SGAs (Itkin et al., 2011; Itkin et al., 2013).

78 The conserved jasmonate (JA) signaling pathway is widely recognized to induce
79 specialized metabolism upon herbivore or necrotrophic pathogen attack in many plant species
80 (De Geyter et al., 2012; Wasternack and Strnad, 2019). The oxylipin-derived JA hormone
81 regulates the expression of its target genes by controlling the activity of certain transcription
82 factors (TFs) that belong to, among others, the basic helix-loop-helix (bHLH) family (Goossens

83 et al., 2017). At low intracellular concentrations of the bioactive (+)-7-*iso*-jasmonoyl-L-
84 isoleucine (JA-Ile) (Fonseca et al., 2009), the activity of these TFs is repressed by the JA ZIM
85 DOMAIN (JAZ) proteins (Chini et al., 2007; Thines et al., 2007; Chini et al., 2016). Elevated
86 intracellular JA-Ile concentrations promote the formation of a co-receptor complex between the
87 JAZ proteins and the F-box protein CORONATINE INSENSITIVE1 (COI1) (Yan et al., 2009;
88 Sheard et al., 2010; Yan et al., 2018), which leads to the ubiquitination and subsequent
89 proteasomal degradation of the interacting JAZ protein (Chini et al., 2007; Thines et al., 2007).
90 This releases the TFs from repression and is followed by the concerted upregulation of multiple
91 genes involved in the same species-specific specialized metabolic pathway (De Geyter et al.,
92 2012; Wasternack and Strnad, 2019).

93 In tomato and potato, the JA-regulated TF GAME9, which belongs to the
94 APETALA2/Ethylene Response Factor (AP2/ERF) family and is also known as JA-
95 RESPONSIVE ERF 4 (JRE4), is involved in the co-regulation of SGA and cholesterol
96 biosynthesis (Cárdenas et al., 2016; Thagun et al., 2016; Nakayasu et al., 2018). At least for a
97 subset of the cholesterol and SGA biosynthesis genes, GAME9 appears to control their
98 expression in cooperation with tomato MYC2, an ortholog of *Arabidopsis thaliana* (*Arabidopsis*)
99 MYC2, with GAME9 and MYC2 recognizing GC-rich and G-box elements, respectively, in their
100 target promoters (Cárdenas et al., 2016; Thagun et al., 2016). *Arabidopsis* MYC2 and its
101 orthologs in other species, including tomato MYC2, are members of the clade IIIe bHLH TFs,
102 which take on a central role in the JA signaling cascade across the plant kingdom (Boter et al.,
103 2004; Kazan and Manners, 2013; Du et al., 2017; Goossens et al., 2017). Besides MYC2, tomato
104 possesses a second clade IIIe bHLH TF, MYC1, which was shown to regulate mono- and
105 sesquiterpene biosynthesis in the type VI glandular trichomes of tomato leaves and stems
106 (Spyropoulou et al., 2014; Xu et al., 2018), but that has not been implicated in the regulation of
107 SGA biosynthesis yet.

108 In this study, we demonstrate that tomato MYC2, together with its homolog MYC1, is
109 instrumental for the basal, but high SGA content in tomato organs. These two clade IIIe bHLH
110 TFs ensure constitutive SGA biosynthesis by redundantly controlling basal expression of both
111 cholesterol and SGA pathway genes. Although MYC-regulated specialized metabolism is
112 typically induced following an elevation in intracellular JA-Ile levels, we found that the
113 constitutive production of SGAs only partially relies on COI1-dependent JA signaling. Finally,

114 CRISPR-Cas9(VQR) genome editing of an endogenous G-box, which is targeted by MYC1/2, in
115 the promoter of the cholesterol biosynthesis gene *STEROL C-5(6) DESATURASE 2 (C5-SD2)*
116 leads to decreased constitutive expression of *C5-SD2*. This further supports the role of the tomato
117 clade IIIe bHLH TFs in the regulation of constitutive SGA biosynthesis.

118 **RESULTS**

119 **MYC2 Coordinates Constitutive Expression of SGA Biosynthesis Genes**

120 To enhance our understanding of MYC2 as a regulator of SGA production, we consulted
121 previously published RNA-seq data from wild-type and *MYC2-RNAi* seedlings (cultivar M82)
122 (Du et al., 2017). Among the differentially expressed genes (DEGs) between mock-treated wild-
123 type and *MYC2-RNAi* plants (false discovery rate [FDR]-adjusted P value < 0.05), we
124 encountered genes known to be involved in the biosynthesis of SGAs and their precursors (Table
125 1). Indeed, transcript levels of genes encoding enzymes for cycloartenol, cholesterol, and SGA
126 biosynthesis were all significantly reduced in mock-treated *MYC2-RNAi* seedlings compared with
127 wild-type seedlings (Table 1), indicating that MYC2 helps ensure their basal expression.

128 To further investigate the potential role of MYC2 as a transcriptional activator of
129 constitutive SGA biosynthesis, we generated three independent *myc2* loss-of-function hairy root
130 lines (cultivar MoneyMaker) using Clustered Regularly Interspaced Short Palindromic Repeats
131 (CRISPR)-CRISPR associated protein 9 (CRISPR-Cas9) genome editing (Figure 1A and
132 Supplemental Figure 1). We selected nine genes from the list of DEGs involved in SGA
133 biosynthesis (Table 1) and measured their expression by quantitative real-time PCR (qPCR) in
134 mock- and JA-treated control and *myc2* lines. Expression of several of these genes was
135 significantly reduced in mock-treated *myc2* hairy root lines compared with control lines (Figure
136 1B) while their JA inducibility, however, remained intact (Supplemental Figure 2). Expression of
137 *GAME9*, a known regulator of SGA biosynthesis, was comparable between mock-treated control
138 and *myc2* root lines (Figure 1B). Targeted metabolite profiling showed there was only a small
139 decrease in α -tomatine and dehydrotomatine levels between control and *myc2* hairy roots in both
140 mock- and JA-treated conditions (Figure 1C). We did not observe a significant increase of these
141 SGAs upon JA induction, in our hands, neither in control lines nor in *myc2* lines. Taken together,
142 these observations suggest that besides MYC2, another JA-regulated TF is involved in the
143 regulation of constitutive SGA production.

144 For comparison, we measured the expression of a gene involved in JA signaling, *JAZ1*
145 (Sun et al., 2011), and a gene involved in the biosynthesis of polyamines, *ORNITHINE*
146 *DECARBOXYLASE* (*ODC*) (Acosta et al., 2005), which were previously reported to be
147 upregulated upon JA treatment (Chen et al., 2006; Chini et al., 2017). In contrast to the
148 downregulation of several SGA pathway genes in mock-treated *myc2* lines (Figure 1B), the
149 expression of *JAZ1* and *ODC* was not reduced in mock-treated *myc2* lines compared to control
150 lines (Figure 2A). The JA inducibility of *JAZ1* and *ODC* transcription in *myc2* hairy roots was
151 not affected either (Figure 2A). Next, we determined the levels of two phenylpropanoid-
152 polyamine conjugates, tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine, which are both
153 reported to be highly JA-inducible compounds (Chen et al., 2006). While the levels of two main
154 SGAs were decreased between mock-treated control and *myc2* hairy roots (Figure 1C), the levels
155 of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine were not (Figure 2B). JA-treated *myc2*
156 lines, however, accumulated less of these polyamines than JA-treated control lines did (Figure
157 2B). These results suggest that MYC2 specifically regulates the constitutive biosynthesis of
158 SGAs and not of other specialized metabolites such as the phenylpropanoid-polyamine
159 conjugates. Conversely, MYC2 is clearly involved, together with other, yet elusive, TFs, in the
160 induction of phenylpropanoid-polyamine conjugate biosynthesis upon JA signaling.

161 **Tomato Has Two bHLH Clade IIIe Family Members**

162 The intact JA inducibility but reduced basal expression of SGA pathway genes and the slight
163 decrease of two main SGAs in *myc2* hairy root lines (Supplemental Figure 2 and Figure 1B–C)
164 suggested that another JA-regulated TF is involved in the regulation of SGA biosynthesis. In
165 Arabidopsis, bHLH clade IIIe consists of MYC2 and three other MYC TFs that all contribute to
166 the JA response (Goossens et al., 2017). Therefore, using the PLAZA comparative genomics
167 platform (Van Bel et al., 2018), we searched for other tomato orthologs of Arabidopsis MYC2
168 and only identified MYC1, a regulator of type VI glandular trichome development and volatile
169 terpene biosynthesis within trichomes (Spyropoulou et al., 2014; Xu et al., 2018). Phylogenetic
170 analysis revealed that duplication of an Arabidopsis *MYC2* ortholog must have occurred at the
171 base of the Solanaceae and that both copies were retained in the *S. lycopersicum* genome (Figure
172 3A) as is the case with several other Solanaceae species (Xu et al., 2017). While the Arabidopsis
173 bHLH clade IIIe consists of MYC2, MYC3, MYC4, and MYC5, it appears to be limited to only

174 two members in Solanaceae species (Figure 3A). A survey of public transcriptome data (cultivar
175 Heinz 1706) (Zouine et al., 2017) showed that the two tomato clade IIIe bHLH members, *MYC1*
176 and *MYC2*, exhibit a similar expression pattern (Figure 3B). In addition, transcript levels of both
177 of these TFs were increased upon JA treatment of control hairy roots (Figure 3C). Although
178 *MYC1* and *MYC2* may have distinct roles, such as in the regulation of glandular trichome
179 development and volatile terpene biosynthesis, which specifically involves *MYC1* but not *MYC2*
180 (Spyropoulou et al., 2014; Xu et al., 2018), our data indicate that *MYC1* and *MYC2* might have
181 overlapping functions as well.

182 ***MYC1* and *MYC2* Redundantly Control SGA Biosynthesis**

183 Previously, we reported that *MYC2* and *GAME9* act synergistically to transactivate the promoter
184 of *C5-SD2*, a gene involved in cholesterologenesis, when fused to the *FIREFLY LUCIFERASE*
185 (*fluc*) gene in tobacco (*Nicotiana tabacum*) protoplasts (Cárdenas et al., 2016). Here, we show
186 that a similar cooperative action can be observed between *MYC1* and *GAME9* (Figure 4A). A G-
187 box present within the *C5-SD2* promoter was formerly found to be bound by *MYC2* and to be
188 essential for the transactivation of this promoter by the combined action of *MYC2* and *GAME9*
189 (Cárdenas et al., 2016). Mutating that same G-box in a 333-bp *C5-SD2* promoter region, which
190 was shown to be sufficient for synergistic transactivation by *MYC1/MYC2* and *GAME9* (Figure
191 4B), led to severely reduced induction of the luciferase activity (Figure 4C), while no luciferase
192 induction was measured for a promoter deletion construct lacking the G-box (Figure 4C). Thus,
193 these data indicate that *MYC1* might play a similar role as *MYC2* in regulating SGA biosynthesis
194 by binding the G-box in the promoters of SGA pathway genes and activating their expression in
195 synergy with *GAME9*.

196 To explore whether *MYC1* is indeed a regulator of SGA production *in planta*, we
197 generated three independent *myc1* loss-of-function hairy root lines (cultivar Moneymaker) using
198 CRISPR-Cas9 genome editing (Figure 5A and Supplemental Figure 3). A qPCR analysis of
199 cycloartenol, cholesterol, and SGA biosynthesis genes revealed that some of them were
200 significantly downregulated in mock-treated *myc1* hairy roots (Figure 5B), whereas no effect was
201 observed on their JA inducibility (Supplemental Figure 4). We checked if the lack of JA-
202 inducible effects in *myc1* and *myc2* single mutants could be explained by a genetic compensation
203 response (Ma et al., 2019), but we did not observe an upregulation of *MYC2* in *myc1* lines or vice

204 versa (Supplemental Figure 5). Levels of α -tomatine and dehydrotomatine were not affected in
205 *myc1* lines compared with control lines, neither in mock- nor in JA-treated conditions (Figure
206 5C). The expression of *JAZ1* and *ODC* was unaltered in both mock- and JA-treated *myc1* hairy
207 roots compared to control hairy roots (Figure 6A) and the levels of tris(dihydrocaffeoyl)spermine
208 and *N*-caffeoylputrescine were only reduced between JA-treated control and *myc1* root lines
209 (Figure 6B). These results are comparable to those observed for *myc2* lines (Figure 1–2),
210 indicating that MYC1 and MYC2 might have redundant roles in regulating the constitutive
211 production of SGAs and their precursors.

212 Next, we used CRISPR-Cas9 genome editing to target *MYC1* and *MYC2* simultaneously,
213 which yielded one double *myc1 myc2* hairy root knockout line (Supplemental Figure 6). We
214 measured the expression of both SGA and more upstream biosynthesis genes and observed
215 severely reduced transcript levels for most of them in mock-treated *myc1 myc2* hairy roots
216 compared with control hairy roots (Figure 7A), while no decrease was detected in the expression
217 of *JAZ1* and *ODC* (Figure 8A). Upon JA treatment, the transcription of not only cholesterol and
218 SGA biosynthesis genes but also of *JAZ1* and *ODC* was not induced or not as strongly induced
219 anymore in the *myc1 myc2* line compared with the control lines (Supplemental Figure 7 and
220 Figure 8A). In accordance with these observations, the *myc1 myc2* line exhibited a 60–90%
221 decrease in α -tomatine and dehydrotomatine content in both mock- and JA-treated conditions
222 (Figure 7B). Although the biosynthesis of tris(dihydrocaffeoyl)spermine and *N*-
223 caffeoylputrescine was no longer induced by JA in *myc1 myc2* hairy roots, their basal levels were
224 not significantly reduced in *myc1 myc2* hairy roots compared to control hairy roots (Figure 8B).
225 Thus, SGA pathway genes appear to be specifically affected while genes involved in canonical
226 JA signaling and the biosynthesis of phenylpropanoid-polyamine conjugates are not. Taken
227 together, these data demonstrate that MYC1 and MYC2 are functionally redundant in specifically
228 controlling the constitutive biosynthesis of SGAs.

229 **SGA Biosynthesis Partially Depends on COI1-Mediated JA Signaling**

230 The primary JA signaling pathway is highly conserved in the plant kingdom (Chini et al., 2016)
231 and is initiated by the perception of JA-Ile by a co-receptor complex consisting of the F-box
232 protein COI1 and a JAZ repressor (Yan et al., 2009; Sheard et al., 2010; Yan et al., 2018).
233 Subsequent proteasomal degradation of the interacting JAZ protein (Chini et al., 2007; Thines et

234 al., 2007) releases JA-regulated TFs from repression by JAZ (Chini et al., 2007; Thines et al.,
235 2007; Chini et al., 2016). As in all investigated plant species, also in tomato the JAZ proteins can
236 interact with MYC2 and, thereby, repress the transcription of MYC2-regulated genes (Du et al.,
237 2017). Accordingly, COI1-dependent signaling has been suggested to be essential for JA-induced
238 upregulation of SGA pathway genes (Abdelkareem et al., 2017). To investigate whether COI1
239 activity is also involved or required for the constitutive production of SGAs, we generated three
240 independent *coi1* loss-of-function hairy root lines (cultivar MoneyMaker) using CRISPR-Cas9
241 genome editing (Figure 9A and Supplemental Figure 8). Basal expression of not only several
242 SGA pathway genes but also of *JAZ1* and *ODC* was significantly reduced in *coi1* hairy roots
243 compared with control hairy roots (Figure 9B and Figure 10A). As expected, the expression of
244 SGA biosynthesis genes, *JAZ1*, and *ODC* was not induced when *coi1* lines were treated with JA
245 (Supplemental Figure 9 and Figure 10A). Furthermore, the α -tomatine and dehydrotomatine
246 content in *coi1* hairy roots was decreased by 30–50% compared with control lines in both JA-
247 and mock-treated conditions (Figure 9C), while tris(dihydrocaffeoyl)spermine and *N*-
248 caffeoylputrescine levels were only reduced in JA-treated conditions (Figure 10B). This evidence
249 suggests a partial dependence of constitutive SGA production on COI1-dependent signaling,
250 possibly due to the stabilization and accumulation of JAZ proteins that block MYC1/2 activity.

251 **Genome Editing of a G-Box Decreases Constitutive *C5-SD2* Expression**

252 In all investigated mutant genotypes (*myc1*, *myc2*, *myc1 myc2*, and *coi1*), the cholesterologenesis
253 gene *C5-SD2* showed the strongest decrease in gene expression (Figure 1B, 5B, 7A, and 9B).
254 Transient expression assays in tobacco protoplasts showed that a G-box present within the *C5-SD2*
255 promoter is necessary for the transactivation of this promoter by MYC1 or MYC2 in
256 combination with GAME9 (Figure 4) (Cárdenas et al., 2016). To investigate the relevance of this
257 G-box *in planta*, we decided to target this *cis*-regulatory element in tomato hairy roots by genome
258 editing using an engineered version of Cas9 that recognizes 5'-NGA-3' as protospacer adjacent
259 motif (PAM) (Kleinstiver et al., 2015). Three independent hairy root lines, denominated *g* lines,
260 were generated in which the G-box motif was either deleted or disrupted. Line *g*^{#1} contains a 33-
261 bp deletion removing the G-box, while *g*^{#2} and *g*^{#3} carry a thymidine insertion and a 2-bp deletion
262 within the G-box, respectively (Figure 11A). The 2-bp deletion in line *g*^{#3} creates an alternative
263 G-box (5'-CACGTT-3'). The transcript level of *C5-SD2* was significantly reduced in both mock-

264 and JA-treated *g* lines compared with control lines, with the exception of mock-treated *g*^{#3} hairy
265 roots (Figure 11B). However, the JA-induced upregulation of *C5-SD2* in all three *g* lines was
266 comparable to that in control hairy roots (Supplemental Figure 10), suggesting that the observed
267 decrease in *C5-SD2* transcript levels in JA-treated conditions is merely due to the reduced basal
268 *C5-SD2* expression. Next, we cloned *C5-SD2* promoter fragments from the *g* lines and fused
269 them to the *fLUC* gene to create reporter constructs corresponding to the genome edited *C5-SD2*
270 promoters. Transient expression assays in tobacco protoplasts showed that MYC2 and GAME9
271 were unable to transactivate these genome edited *C5-SD2* promoter fragments in which the G-
272 box was either removed or disrupted (Figure 11C).

273 The expression levels of other cholesterol and most SGA biosynthesis genes were not
274 altered in mock-treated *g* lines (Figure 11B), indicating that the effect on *C5-SD2* expression was
275 specific and had no general feedback effects on other cholesterol and SGA pathway genes.
276 Nonetheless, we observed a small but significant decrease in dehydrotomatine levels compared
277 with control lines (Figure 11D), indicating that targeting of specific and essential *cis*-regulatory
278 elements in promoters of key pathway genes is sufficient to alter metabolic fluxes in pathways
279 and, thereby, modulate metabolite levels. For comparison, we measured the α -tomatine and
280 dehydrotomatine content in two independent *c5-sd2* loss-of-function hairy root lines (cultivar
281 Moneymaker) obtained by CRISPR-Cas9 genome editing (Supplemental Figure 11A). The levels
282 of the main SGAs were reduced by approximately 50% in both mock- and JA-treated conditions
283 compared with control lines (Supplemental Figure 11B). These results support our hypothesis
284 that MYC1 and MYC2 help ensure constitutive *C5-SD2* expression by binding a G-box in the
285 *C5-SD2* promoter.

286 DISCUSSION

287 Throughout the plant kingdom, transcriptional regulators belonging to the clade IIIe bHLH TFs
288 are master regulators of the JA-induced production of specialized metabolites that help plants fend
289 off biotic enemies (De Geyter et al., 2012; Goossens et al., 2017). Here, we show that two tomato
290 clade IIIe bHLH TFs, MYC1 and MYC2, control the constitutive production of SGAs that grant
291 protection against a wide variety of herbivores and pathogens by making up a chemical defense
292 barrier (Friedman, 2002). Accordingly, CRISPR-Cas9-mediated disruption or deletion of an
293 endogenous G-box, which is targeted by MYC1/2, in the promoter of *C5-SD2* leads to decreased

294 basal *C5-SD2* expression. Although the activation of specialized metabolism by MYC TFs is
295 typically initiated by the perception of JA-Ile, constitutive SGA biosynthesis seems to only
296 partially rely upon COI1-dependent signaling.

297 **JA-Regulated TFs Control Constitutive Alkaloid Production in Solanaceous Species**

298 SGAs provide multiple members of the *Solanum* genus constitutive protection against a broad
299 range of herbivores and pathogens (Friedman, 2002). Here, we report that the tomato JA-
300 regulated TFs MYC1 and MYC2 coordinate the basal biosynthesis of these cholesterol-derived
301 products. A double *myc1 myc2* hairy root knockout line displayed suppressed expression of genes
302 known to be involved in the biosynthesis of SGAs and their precursors. In addition, the
303 expression of several SGA pathway genes in *myc1 myc2* root lines was not induced anymore by
304 JA treatment. Although *myc1 myc2* hairy roots no longer exhibited JA-induced upregulation of
305 *ODC*, which encodes an enzyme in the highly JA-inducible polyamine pathway (Chen et al.,
306 2006), the basal expression of *ODC* was unaffected. Accordingly, targeted metabolite profiling
307 showed that *myc1 myc2* hairy roots contained severely reduced constitutive levels of the main
308 tomato SGAs α -tomatine and dehydrotomatine but not of the phenylpropanoid-polyamine
309 conjugates tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine. The levels of the latter
310 compounds in *myc1 myc2* root lines were only affected in JA-treated conditions. Basal
311 transcription of some, but not all, cholesterol and SGA biosynthesis genes was reduced in single
312 *myc1* and *myc2* knockout lines, however, their JA inducibility was retained. Moreover, only a
313 modest decrease in α -tomatine and dehydrotomatine content was observed in *myc2* hairy root
314 lines alone. This indicates that there is functional redundancy between MYC1 and MYC2 in the
315 control of SGA accumulation. Likewise, both constitutive and insect-inducible glucosinolate
316 production in Arabidopsis is redundantly controlled by the clade IIIe bHLH TFs MYC2, MYC3,
317 and MYC4 (Schweizer et al., 2013).

318 Both MYC1 and MYC2 directly regulate the expression of *C5-SD2*, a cholesterologenesis
319 gene, and likely of other cholesterol and SGA biosynthesis genes as well, in synergy with
320 GAME9 by binding G-box and GC-rich elements in their promoters. Disruption or deletion of a
321 G-box in the endogenous promoter of *C5-SD2* by genome editing leads to reduced *C5-SD2*
322 transcription in both mock- and JA-treated conditions, which suggests that the synergistic action
323 of these transcriptional regulators contributes to the basal expression of *C5-SD2*. Like our *myc1*

324 *myc2* hairy root knockout line, tomato plant lines in which *GAME9* is either silenced or mutated
325 display suppressed basal transcript levels of cholesterol and SGA biosynthesis genes (Cárdenas et
326 al., 2016; Nakayasu et al., 2018). Consequently, these lines accumulate less SGAs (Cárdenas et
327 al., 2016; Nakayasu et al., 2018), suggesting that *GAME9*, another JA-regulated TF, regulates the
328 basal expression of genes needed for the constitutive production of SGAs. Interestingly, tobacco
329 *MYC1* and *MYC2* orthologs control the production of nicotine, another constitutively highly
330 accumulating alkaloid, together with *ERF189*, a tobacco JA-regulated AP2-ERF family member
331 related to *GAME9* (Shoji and Hashimoto, 2011). Target gene promoters harbor G-box and GC-
332 rich elements that allow binding of these clade IIIe bHLH TFs and *ERF189*, respectively (Shoji
333 and Hashimoto, 2011; Kajikawa et al., 2017; Xu et al., 2017). Silencing of *MYC1* or *MYC2*
334 orthologs in tobacco leads to suppressed constitutive transcription of genes involved in nicotine
335 biosynthesis and severely reduced basal alkaloid levels (Shoji and Hashimoto, 2011).
336 Furthermore, like *GAME9*, *ERF189* controls alkaloid accumulation in unelicited conditions since
337 *nic1 nic2* hairy roots, in which the most severely repressed AP2/ERF is *ERF189*, display
338 decreased basal expression of nicotine biosynthesis and transport genes as well as declined
339 constitutive alkaloid production (Shoji et al., 2010). This suggests that the role of clade IIIe
340 bHLH TFs in the regulation of constitutive biosynthesis of bioactive specialized metabolites may
341 occur within additional *Solanaceae* species and might even be widespread within the plant
342 kingdom.

343 An important question that remains is how these JA-regulated TFs are able to drive
344 constitutive biosynthesis of highly accumulating alkaloids in *Solanaceae* members. The
345 proximity of G-box and GC-rich elements in their target promoters and the collaborative action
346 of these clade IIIe bHLH and AP2/ERF TFs suggest their cooperative binding, which can be a
347 way to enhance their specificity and binding affinity for *cis*-regulatory elements (CREs)
348 (Brkljacic and Grotewold, 2017). Target specificity of Arabidopsis *MYC2/MYC3/MYC4* has
349 been proposed to be governed by their interaction with R2R3-MYB TFs (Schweizer et al., 2013).
350 Furthermore, competitive binding between these MYBs and the JAZ repressors to the JAZ
351 interaction domain of *MYC2/3/4* has already been forwarded as a mechanism for the regulation
352 of constitutive glucosinolate production in Arabidopsis (Schweizer et al., 2013). Thus, it is
353 possible that tomato *MYC1/2* and *GAME9*, as well as their tobacco counterparts, form protein
354 complexes that may facilitate the shielding of clade IIIe bHLH TFs from JAZ repressors.

355 **Partial Dependence of Constitutive SGA Biosynthesis on JA Signaling**

356 JA signaling provokes transcriptional reprogramming, leading toward the biosynthesis of species-
357 specific defense compounds across the plant kingdom. Although perception of JA-Ile typically
358 promotes fast and strong upregulation of specialized metabolism, in our hands tomato hairy roots
359 that were treated with JA for one day did not display a marked increase in SGA levels whereas
360 they did in the levels of phenylpropanoid-polyamine conjugates. Only after three to four days of
361 continuous JA treatment, we and others were able to observe a modest increase in SGA content
362 of 1.6- to 1.8-fold (Supplemental Figure 12) (Nakayasu et al., 2018), suggesting that this may not
363 be a primary effect of JA signaling. The same holds true for the limited JA-induced upregulation
364 of nicotine biosynthesis in tobacco plants and hairy roots (Shoji et al., 2008). In both tomato and
365 tobacco, COI1-mediated perception of JA-Ile has been suggested to be essential for the minimal
366 increase in alkaloid production upon JA elicitation (Shoji et al., 2008; Abdelkareem et al., 2017).
367 Here, we report that constitutive SGA biosynthesis declines in mock-treated tomato *coil* loss-of-
368 function mutants, which confirms previous observations (Abdelkareem et al., 2017). This might
369 be due to the stabilization and accumulation of JAZ proteins that block the activity of MYC1/2.
370 The decrease in constitutive SGA content in *coil* lines, however, is not as severe as in the double
371 *myc1 myc2* knockout line. Hence, this suggests that the regulation of basal SGA production only
372 partially relies upon COI1-dependent JA signaling and that MYC1 and MYC2, likely together
373 with GAME9, are able to regulate SGA biosynthesis independent of JA signaling as well. The
374 reduction, but not absence, of SGAs in *spr2* tomato plants, in which JA biosynthesis is impaired,
375 further supports this notion (Montero-Vargas et al., 2018).

376 Transcriptional coordination of genes involved in the same specialized metabolic pathway
377 can be accomplished by their promoters acquiring CREs that can be bound by JA-regulated TFs
378 (Mertens et al., 2016; Shoji, 2019). It is therefore plausible to assume that, through the
379 recruitment of G-box and GCC-box elements to the promoters of alkaloid biosynthesis genes, the
380 JA-regulated MYC1/2 and GAME9, and their orthologs in tobacco, evolved to accommodate the
381 constitutive chemical defense barrier made up of alkaloids.

382 **METHODS**

383 **DNA Constructs**

384 *Transient Expression Assay Constructs*

385 For transient expression assays, the coding sequence of tomato *MYC1* was PCR-amplified with
386 the primers listed in Supplemental Table 1 and recombined in a Gateway donor vector
387 (Invitrogen). Subsequently, a Gateway LR reaction (Invitrogen) was performed with the p2GW7
388 vector (Vanden Bossche et al., 2013). The *C5-SD2* promoter regions in which a G-box was
389 disrupted or removed were PCR-amplified from *g* hairy root lines (cultivar Moneymaker) and
390 recombined in a Gateway donor vector (Invitrogen). Next, Gateway LR reactions (Invitrogen)
391 were performed with the pGWL7 vector (Vanden Bossche et al., 2013). All other constructs used
392 for transient expression assays were generated previously (Cárdenas et al., 2016).

393 *CRISPR-Cas9 Constructs*

394 To select CRISPR-Cas9 guide (g)RNA target sites, CRISPOR (<http://crispor.tefor.net/>)
395 (Haeussler et al., 2016) was used, with as PAM requirement 5'-NGA-3' for targeting the G-box
396 in the *C5-SD2* promoter and 5'-NGG-3' for single and double knockouts. CRISPR-Cas9
397 constructs were cloned as previously described (Fauser et al., 2014; Ritter et al., 2017; Pauwels et
398 al., 2018). Briefly, for each gRNA target site, two complementary oligonucleotides with 4-bp
399 overhangs (Supplemental Table 1) were annealed and inserted by a Golden Gate reaction with
400 *BpiI* (Thermo Scientific) and T4 DNA ligase (Thermo Scientific) in following Gateway entry
401 vectors: pEN-C1.1 (Fauser et al., 2014) was used for targeting the G-box in the *C5-SD2* promoter
402 by a single gRNA approach, pMR217 (L1–R5) and pMR218 (L5–L2) (Ritter et al., 2017) were
403 used for single *myc1*, *myc2*, *coi1*, and *c5-sd2* knockouts by a dual gRNA approach, and pMR217
404 (L1–R5) (Ritter et al., 2017), pMR219 (L5–L4), pMR204 (R4–R3), and pMR205 (L5–L2) were
405 used for the double *myc1 myc2* knockout. To allow combining four gRNA modules, primers
406 (Supplemental Table 1) were designed to amplify the gRNA module from pEn-C1.1 (L1–L2)
407 (Fauser et al., 2014) adding appropriate attB/attBr flanking sites (L5–L4, B4r–B3r, and L3–L2) to
408 each fragment. The amplified fragments were then cloned into the corresponding pDONR221
409 vector (pDONR221 P5–P4, P4r–P3r, and P3–P2) by Gateway BP reactions (Invitrogen) to
410 generate entry clones suitable for MultiSite Gateway LR cloning. An additional *BbsI* site in the
411 pDONR backbone was eliminated by site-directed mutagenesis using primers noBbsI_F and
412 noBbsI_R (Supplemental Table 1) followed by an In-Fusion reaction (Takara Bio USA). In order
413 to yield the final binary vectors, (MultiSite) Gateway LR reactions (Invitrogen) were used. One

414 gRNA module was recombined with pDe-Cas9(VQR)-Km (Kleinstiver et al., 2015; Swinnen et
415 al., 2020) to target the G-box in the *C5-SD2* promoter. Two and four gRNA modules were
416 recombined with pDe-Cas9-Km (Ritter et al., 2017) for single *myc1*, *myc2*, *coi1*, and *c5-sd2*
417 knockouts and a double *myc1 myc2* knockout, respectively.

418 **Transient Expression Assays in Tobacco Protoplasts**

419 Transient expression assays in protoplasts prepared from *N. tabacum* Bright Yellow-2 (BY-2)
420 cells were performed as previously described (Vanden Bossche et al., 2013). Briefly, protoplasts
421 were transfected with a *pC5-SD2::fLUC* reporter construct and effector constructs overexpressing
422 *GUS*, *MYC1*, *MYC2*, *GAME9* or a combination thereof. A *pCaMV35S::rLUC* construct was co-
423 transfected for normalization of fLUC activity. Two micrograms of each construct were
424 transfected and total DNA added was equalized with a *pCaMV35S::GUS* control construct. After
425 overnight incubation followed by lysis of the cells, the luciferase activities were measured using
426 the Dual-Luciferase Reporter Assay System (Promega). Each assay was carried out in eight
427 biological repeats. Statistical significance was determined by unpaired Student's *t*-tests.

428 **Generation and Cultivation of Tomato Hairy Roots**

429 *S. lycopersicum* (cultivar Moneymaker) seed sterilization, rhizogenic *Agrobacterium*-mediated
430 transformation of tomato seedlings and cultivation of hairy roots were carried out as previously
431 described (Harvey et al., 2008; Ron et al., 2014) with following modifications. Tomato seeds
432 were surface-sterilized in 70% (v/v) ethanol for 5 min followed by 3% (v/v) NaOCl for 20 min
433 and three washes with sterile water. Seeds were plated on Murashige and Skoog (MS) medium
434 (pH 5.8) containing 4.3 g/L of MS (Duchefa), 0.5 g/L of MES, 10 g/L of sucrose, and 10 g/L of
435 agar (Neogen) in Magenta boxes. Boxes were put in the dark at 4°C for two days, in the dark at
436 24°C for one day, and in a 24°C controlled photoperiodic growth chamber (16:8 photoperiods)
437 for ca. two weeks until cotyledons were fully expanded and the true leaves were just emerged.
438 Competent rhizogenic *Agrobacterium* (strain ATCC15834) cells were transformed by
439 electroporation with the desired binary vector, plated on yeast extract broth (YEB) medium with
440 100 mg/L of spectinomycin, and incubated at 28°C for four days. Each transformed culture was
441 inoculated from plate into liquid YEB medium with 100 mg/L of spectinomycin and incubated
442 overnight at 28°C with shaking at 200 rpm. Each transformed culture was used to transform

443 approximately 40 cotyledon explants. Using a scalpel, cotyledons were cut in half after cutting
444 off their base and top, which was followed by immersion of the explants in *Agrobacterium*
445 culture with an optical density of 0.2–0.3 at 600 nm in liquid MS medium for 20 min. Next, the
446 explants were blotted on sterile Whatman filter paper and transferred with their adaxial side down
447 to plates with MS medium (pH 5.8) containing 4.4 g/L of MS supplemented with vitamins
448 (Duchefa), 0.5 g/L of MES, 30 g/L of sucrose, and 8 g/L of agar (Neogen) without antibiotics.
449 After three to four days of incubation in the dark at 25°C (Oberpichler et al., 2008), the explants
450 were transferred with their adaxial side down to plates with MS medium (pH 5.8) containing 4.4
451 g/L of MS supplemented with vitamins (Duchefa), 0.5 g/L of MES, 30 g/L of sucrose, and 8 g/L
452 of agar (Neogen) with 200 mg/L of cefotaxime and 50 mg/L of kanamycin. These plates were
453 returned to the dark at 25°C until hairy roots emerged from infected sites. Hairy roots were
454 excised and cultured on fresh plates with MS medium (pH 5.8) containing 4.4 g/L of MS
455 supplemented with vitamins (Duchefa), 0.5 g/L of MES, 30 g/L of sucrose, and 10 g/L of agar
456 (Neogen) with 200 mg/L of cefotaxime and 50 mg/L of kanamycin. After three rounds of
457 subculture on plates with MS medium of the same composition, hairy roots were subcultured
458 every four weeks on plates with MS medium without antibiotics for maintenance.

459 **Identification of CRISPR-Cas9 Hairy Root Mutants**

460 CRISPR-Cas9 mutants were identified as described previously (Swinnen et al., 2020). Genomic
461 DNA was prepared from homogenized hairy root cultures using extraction buffer (pH 9.5)
462 containing 0.1 M of tris(hydroxymethyl)aminomethane (Tris)-HCl, 0.25 M of KCl, and 0.01 M
463 of ethylenediaminetetraacetic acid (EDTA). This mixture was incubated at 95°C for 10 min and
464 subsequently cooled at 4°C for 5 min. After addition of 3% (w/v) BSA, collected supernatant was
465 used as a template in a standard PCR reaction using GoTaq (Promega) with Cas9-specific
466 primers or primers to amplify the gRNA(s) target region(s) (Supplemental Table 1). PCR
467 amplicons containing the gRNA(s) target site(s) were purified using HighPrep PCR reagent
468 (MAGBIO). After Sanger sequencing of the purified PCR amplicons with an amplification
469 primer located approximately 200 bp from the Cas9 cleavage site, quantitative sequence trace
470 data were decomposed using Inference of CRISPR Editing (ICE) CRISPR Analysis Tool
471 (<https://ice.synthego.com/#/>).

472 **Gene Expression Analysis by Quantitative Real-Time PCR**

473 Hairy roots were grown for eight days in liquid MS medium (pH 5.8) containing 4.4 g/L of MS
474 supplemented with vitamins (Duchefa), 0.5 g/L of MES, and 30 g/L of sucrose. Three biological
475 replicates per line were treated for 24 h with 50 μ M of JA or an equal amount of ethanol by
476 replacement of the medium. Hairy roots were rinsed with purified water, harvested by flash
477 freezing in liquid nitrogen, and ground using the Mixer Mill 300 (Retch).

478 Messenger RNA was extracted from approximately 15 mg of homogenized tissue as
479 reported previously (Townsend et al., 2015) with following modifications. Tissue was lysed using
480 500 μ L of lysate binding buffer (LBB) containing 100 mM of Tris-HCl (pH 7.5), 500 mM of
481 LiCl, 10 mM of EDTA (pH 8.0), 1% of sodium dodecyl sulfate (SDS), 5 mM of dithiothreitol
482 (DTT), 15 μ L/mL of Antifoam A, and 5 μ L/mL of 2-mercaptoethanol, and the mixture was
483 allowed to stand for 10 min. Messenger RNA was separated from 200 μ L of lysate using 1 μ L of
484 12.5 μ M of 5' biotinylated polyT oligonucleotide (5'-biotin-
485 ACAGGACATTCGTCGCTTCCTTTTTTTTTTTTTTTTTTTT-3') and the mixture was allowed
486 to stand for 10 min. Next, captured messenger RNA was isolated from the lysate by adding 20 μ L
487 of LBB-washed streptavidin-coated magnetic beads (New England Biolabs) and was allowed to
488 stand for 10 min. Samples were placed on a MagWell Magnetic Separator 96 (EdgeBio) and
489 washed with 200 μ L of washing buffer A (10 mM of Tris-HCl (pH 7.5), 150 mM of LiCl, 1 mM
490 of EDTA (pH 8.0), 0.1% of SDS), washing buffer B (10 mM of Tris-HCl (pH 7.5), 150 mM of
491 LiCl, 1 mM of EDTA (pH 8.0)), and low-salt buffer (20 mM of Tris-HCl (pH 7.5), 150 mM of
492 NaCl, 1 mM of EDTA (pH 8.0)), which were pre-chilled on ice. Elution of messenger RNA was
493 done by adding 20 μ L of 10 mM of Tris-HCl (pH 8.0) with 1 mM of 2-mercaptoethanol followed
494 by incubation of the mixture at 80°C for 2 min.

495 First-strand complementary DNA was synthesized from 20 μ L of messenger RNA eluate
496 by qScript cDNA Synthesis Kit (Quantabio). For control samples, cDNA of three biological
497 replicates was pooled per independent line and treatment. Quantitative real-time PCR (qPCR)
498 reactions were carried out with a LightCycler 480 System (Roche) using Fast SYBR Green
499 Master Mix (Applied Biosystems) and primers (Supplemental Table 1) designed by QuantPrime
500 (<https://www.quantprime.de/>) (Arvidsson et al., 2008). Gene expression levels were quantified
501 relative to *CLATHRIN ADAPTOR COMPLEXES MEDIUM SUBUNIT (CAC)* and *TAP42-*
502 *INTERACTING PROTEIN (TIP41)* using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

503 Statistical significance of log₂-transformed data was determined by ANOVA followed by Tukey
504 post-hoc analysis ($P < 0.05$).

505 **Targeted Metabolite Profiling by Liquid Chromatography-Mass Spectrometry**

506 Hairy roots were grown for four weeks in liquid MS medium (pH 5.8) containing 4.4 g/L of MS
507 supplemented with vitamins (Duchefa), 0.5 g/L of MES, and 30 g/L of sucrose. Five biological
508 replicates per line were treated for 24 h or 96 h with 50 μ M of JA or an equal amount of ethanol
509 by replacement of the medium. Hairy roots were rinsed with purified water, harvested by flash
510 freezing in liquid nitrogen, and ground with pestle and mortar. Approximately 400 mg of
511 homogenized tissue was extracted using 1 mL of MeOH at room temperature for 10 min. Next,
512 supernatant was evaporated to dryness under vacuum, the residue was dissolved in 800 μ L of
513 H₂O/cyclohexane (1:1, v/v), and 100 μ L of the aqueous phase was filtered using a 0.2 μ m filter
514 plate (Pall) and retained for analysis.

515 For LC-MS, 10 μ L of the sample was injected into an Acquity UPLC BEH C18 column
516 (2.1 x 150 mm, 1.7 μ m) mounted on a Waters Acquity UPLC system coupled to a SYNAPT
517 HDMS Q-TOF via an electrospray ionization source operated negative mode. The following
518 gradient was run using acidified (0.1% (v/v) formic acid) solvents A (water/acetonitrile, 99:1,
519 v/v) and B (acetonitrile/water; 99:1, v/v) at a flow rate of 350 μ L/min: time 0 min, 5% B; 30 min,
520 50% B; 33 min, 100% B. Negative mode MS and chromatogram integration and alignment using
521 the Progenesis QI software package (Waters) were carried out as described (Vanholme et al.,
522 2013). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
523 ($P < 0.05$) or by unpaired Student's *t*-tests.

524 **Phylogenetic Analysis**

525 Amino acid sequences from *A. thaliana* MYC2 orthologs were retrieved from the comparative
526 genomics resource PLAZA 4.0 Dicots (<http://bioinformatics.psb.ugent.be/plaza/>) (Van Bel et al.,
527 2018), with the exception of *N. tabacum* sequences that were retrieved through a BLASTP search
528 in the National Center for Biotechnology Information (NCBI) GenBank protein database. All
529 phylogenetic analyses were conducted in MEGA7 (Kumar et al., 2016). A multiple sequence
530 alignment of the full-length proteins was generated with MUSCLE and can be found in
531 Supplemental Figure 13. Using the Find Best DNA/Protein Models (ML) tool, the Maximum

532 Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 2008) was chosen to
533 infer the phylogenetic tree. Evolutionary rate differences among sites were modeled using a
534 discrete Gamma distribution. All positions with less than 95% site coverage were eliminated.
535 Bootstrap analysis was carried out with 1,000 replicates.

536 Accession Numbers

537 Sequence data from this article can be found in the EMBL/GenBank/Solgenomics data libraries
538 under the following accession numbers: *MYC2* (Solyc08g076930), *MYC1* (Solyc08g005050),
539 *CAS* (Solyc04g070980), *3bHSD2* (Solyc02g081730), *CPI* (Solyc12g098640), *CYP51*
540 (Solyc01g008110), *C14-R* (Solyc09g009040), *8,7-SI* (Solyc06g082980), *SSR2*
541 (Solyc02g069490), *SMO3* (Solyc01g091320), *SMO4* (Solyc06g005750), *C5-SD2*
542 (Solyc02g086180), *7-DR2* (Solyc06g074090), *GAME11* (Solyc07g043420), *GAME6*
543 (Solyc07g043460), *GAME4* (Solyc12g006460), *GAME12* (Solyc12g006470), *GAME25*
544 (Solyc01g073640), *GAME1* (Solyc07g043490), *GAME17* (Solyc07g043480), *GAME18*
545 (Solyc07g043500), *GAME9* (Solyc01g090340), *CAC* (Solyc08g006960), *TIP41*
546 (Solyc10g049850), *JAZ1* (Solyc12g009220), *ODC* (Solyc04g082030), and *COII*
547 (Solyc05g052620).

548 Supplemental Data

549 **Supplemental Figure 1.** Schematic representation of *MYC2* with location of the CRISPR-Cas9
550 cleavage sites and *myc2* mutant sequences.

551 **Supplemental Figure 2.** Unaffected JA inducibility of SGA biosynthesis gene expression in
552 *myc2* lines.

553 **Supplemental Figure 3.** Schematic representation of *MYC1* with location of the CRISPR-Cas9
554 cleavage sites and *myc1* mutant sequences.

555 **Supplemental Figure 4.** Unaffected JA inducibility of SGA biosynthesis gene expression in
556 *myc1* lines.

557 **Supplemental Figure 5.** *myc2* and *myc1* lines do not exhibit upregulated expression of *MYC1*
558 and *MYC2*, respectively.

559 **Supplemental Figure 6.** Schematic representation of *MYC1* and *MYC2* with location of the
560 CRISPR-Cas9 cleavage sites and *myc1 myc2* mutant sequences.

561 **Supplemental Figure 7.** Reduced or absent JA inducibility of SGA biosynthesis gene expression
562 in a *myc1 myc2* line.

563 **Supplemental Figure 8.** Schematic representation of *COI1* with location of the CRISPR-Cas9
564 cleavage sites and *coil* mutant sequences.

565 **Supplemental Figure 9.** SGA biosynthesis gene expression is no longer JA inducible in *coil*
566 lines.

567 **Supplemental Figure 10.** JA inducibility of *C5-SD2* expression is not affected in G-box (*g*)
568 mutant lines.

569 **Supplemental Figure 11.** Reduced SGA levels in *c5-sd2* lines.

570 **Supplemental Figure 12.** Limited SGA induction upon 96h of JA treatment.

571 **Supplemental Figure 13.** Protein alignment generated by MUSCLE used for the phylogenetic
572 tree in Figure 3A.

573 **Supplemental Table 1.** Oligonucleotides used in this study

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583 **AUTHOR CONTRIBUTIONS**

584 G.S., L.P., and A.G. designed the experiments. G.S., M.D.M., J.P., F.J.M.H., E.C., R.D.C.,
585 R.V.B., P.F.C., and M.R. performed experiments. G.S., J.P., L.P., and A.G. analyzed the data.
586 G.S. wrote the article and J.P., L.P., and A.G. complemented the writing. A.G. agrees to serve as
587 the author responsible for contact and ensures communication. All scientists who have
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591 to the list of authors and the identified contributions.

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750

751

752 **FIGURE LEGENDS**

753 **Figure 1.** MYC2 coordinates constitutive SGA biosynthesis in tomato. (A) Schematic
754 representation of *MYC2* with location of the CRISPR-Cas9 cleavage sites. The dark grey box
755 represents the exon. Cas9 cleavage sites for two guide RNAs are indicated with arrowheads. (B)
756 Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed
757 by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2* lines
758 (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control
759 samples, cDNA of three biological replicates was pooled per independent line and treatment.
760 Bars represent mean \log_2 -transformed fold changes relative to the mean of three independent
761 mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-
762 treated (■) values are shown. Statistical significance was determined by ANOVA followed by
763 Tukey post-hoc analysis (P<0.05; indicated by different letters). (C) Relative accumulation of α -
764 tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing
765 *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of
766 JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of
767 mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-
768 treated (■) values are shown. Statistical significance was determined by ANOVA followed by
769 Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL*
770 *SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-*
771 *14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -
772 *glucuronidase*.

773 **Figure 2.** MYC2 helps ensure JA-induced polyamine production in tomato. (A) Relative
774 expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing
775 *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of
776 JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was
777 pooled per independent line and treatment. Bars represent mean \log_2 -transformed fold changes
778 relative to the mean of three independent mock-treated control lines. Error bars denote standard
779 error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance
780 was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different
781 letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine

782 analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2*
783 lines (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars
784 represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote
785 standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical
786 significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated
787 by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

788 **Figure 3.** Tomato has two clade IIIe bHLH family members. (A) Phylogenetic analysis of
789 *Arabidopsis thaliana* and Solanaceae bHLH clade IIIe members using MUSCLE and the
790 Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the
791 number of substitutions per site. The analysis involved amino acid sequences from *Capsicum*
792 *annuum* (CAN.G1216.16, CAN.G298.8), *Solanum tuberosum* (PGSC0003DMG400001161,
793 PGSC0003DMG400017535), *S. lycopersicum* (Solyc08g005050.2, Solyc08g076930.1.1),
794 *Petunia axillaris* (Peaxi162Scf00045g00011, Peaxi162Scf00460g00011), *Nicotiana tabacum*
795 (NP_001312938.1, NP_001311866.1, NP_001312960.1, NP_001313001.1), and *A. thaliana*
796 (AT1G32640, AT5G46830, AT5G46760, AT4G17880). The *A. thaliana* bHLH clade IVa
797 member bHLH25 (AT4G37850) was used to root the tree. Numbers shown are bootstrap values
798 in percentages (based on 1,000 replicates). Evolutionary analyses were conducted in MEGA7
799 (Kumar et al., 2016). (B) Normalized *MYC1* and *MYC2* expression profiles in different organs
800 and developmental stages (cultivar Heinz 1706). Expression data were obtained from
801 TomExpress (Zouine et al., 2017). (C) Relative expression of *MYC1* and *MYC2* analyzed by
802 qPCR. Control hairy root lines expressing *pCaMV35S::GUS* were treated for 24 h with 50 μ M of
803 JA or an equal amount of ethanol. The cDNA of three biological replicates was pooled per
804 independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the
805 mean of three independent mock-treated control lines. Error bars denote standard error (n=3).
806 Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was
807 determined by unpaired Student's *t*-tests (**, P<0.01). Abbreviations: 1 cm, 1 cm immature green
808 fruit; 2 cm, 2 cm immature green fruit; 3 cm, 3 cm immature green fruit; MG, mature green fruit;
809 BR, breaker fruit; RR, red ripe (breaker + 10 days) fruit.

810 **Figure 4.** MYC1 and MYC2 transactivate the promoter of *C5-SD2* together with GAME9. (A-C)
811 Tobacco BY-2 protoplasts were transfected with a *pC5-SD2(1549 bp)::fLUC* (A), *pC5-SD2(333*

812 *bp*::*fLUC* (B), *pC5-SD2*(333 *bp* with mutated *G-box*::*fLUC* or *pC5-SD2*(207 *bp* without *G-*
813 *box*::*fLUC* (C) reporter construct and effector constructs overexpressing *MYC1*, *MYC2*, *GAME9*
814 or a combination thereof. A *pCaMV35S*::*rLUC* construct was co-transfected for normalization of
815 *fLUC* activity. Bars represent mean fold changes relative to the mean of protoplasts transfected
816 with a *pCaMV35S*::*GUS* control construct (grey bar). Dashed lines represent the 2-fold cut off for
817 promoter transactivation. Error bars denote standard error (n = 8). Statistical significance was
818 determined by unpaired Student's *t*-tests (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).
819 Abbreviations: BY-2, Bright Yellow-2; *pC5-SD2*, promoter of *C5-SD2*; *GUS*, β -glucuronidase.

820 **Figure 5.** *MYC1* regulates constitutive expression of SGA biosynthesis genes in tomato. (A)
821 Schematic representation of *MYC1* with location of the CRISPR-Cas9 cleavage sites. The dark
822 grey box represents the exon and light grey boxes represent the UTRs. Cas9 cleavage sites for
823 two guide RNAs are indicated with arrowheads. (B) Relative expression of cholesterologenesis
824 genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy root lines
825 expressing *pCaMV35S*::*GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50
826 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological
827 replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed
828 fold changes relative to the mean of three independent mock-treated control lines. Error bars
829 denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown.
830 Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P <
831 0.05; indicated by different letters). (C) Relative accumulation of α -tomatine and
832 dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S*::*GUS*
833 (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount
834 of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error
835 bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown.
836 Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P
837 <0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE*
838 *2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-*
839 *DEHYDROCHOLESTEROL REDUCTASE 2*; UTR, untranslated region; *GUS*, β -glucuronidase.

840 **Figure 6.** *MYC1* helps ensure JA-induced polyamine biosynthesis in tomato. (A) Relative
841 expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing

842 *pCaMV35S::GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA
843 or an equal amount of ethanol. For control samples, cDNA of three biological replicates was
844 pooled per independent line and treatment. Bars represent mean \log_2 -transformed fold changes
845 relative to the mean of three independent mock-treated control lines. Error bars denote standard
846 error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance
847 was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different
848 letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine
849 analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc1*
850 lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars
851 represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote
852 standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical
853 significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated
854 by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

855 **Figure 7.** MYC1 and MYC2 redundantly regulate constitutive SGA biosynthesis in tomato. (A)
856 Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed
857 by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2*
858 (*myc12*) line (green bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol.
859 For control samples, cDNA of three biological replicates was pooled per independent line and
860 treatment. Bars represent mean \log_2 -transformed fold changes relative to the mean of three
861 independent mock-treated control lines. Error bars denote standard error (n=3). Individual mock-
862 (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA
863 followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (B) Relative
864 accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines
865 expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2 (myc12)* line (green bars) were treated
866 for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes
867 relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n = 5). Individual
868 mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by
869 ANOVA followed by Tukey post-hoc analysis (P < 0.05; indicated by different letters).
870 Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL*

871 *OXIDASE 3; C14-R, STEROL C-14 REDUCTASE; 7-DR2, 7-DEHYDROCHOLESTEROL*
872 *REDUCTASE 2; GUS, β -glucuronidase.*

873 **Figure 8.** MYC1 and MYC2 redundantly regulate JA-induced polyamine accumulation in
874 tomato. (A) Relative expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines
875 expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2 (myc12)* line (green bars) were treated
876 for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three
877 biological replicates was pooled per independent line and treatment. Bars represent mean log₂-
878 transformed fold changes relative to the mean of three independent mock-treated control lines.
879 Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are
880 shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
881 (P<0.05; indicated by different letters). (B) Relative accumulation of
882 tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine analyzed by LC-MS. Control hairy root
883 lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2 (myc12)* line (green bars) were
884 treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold
885 changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5).
886 Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was
887 determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different
888 letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

889 **Figure 9.** Constitutive SGA biosynthesis partially depends on COI1-mediated JA signaling. (A)
890 Schematic representation of *COI1* with location of the CRISPR-Cas9 cleavage sites. Dark grey
891 boxes represent exons, solid lines represent introns, and light grey boxes represent UTRs. Cas9
892 cleavage sites for two guide RNAs are indicated with arrowheads. (B) Relative expression of
893 cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy
894 root lines expressing *pCaMV35S::GUS* (grey bars) and *coi1* lines (pink bars) were treated for
895 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three
896 biological replicates was pooled per independent line and treatment. Bars represent mean log₂-
897 transformed fold changes relative to the mean of three independent mock-treated control lines.
898 Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are
899 shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
900 (P < 0.05; indicated by different letters). (C) Relative accumulation of α -tomatine and

901 dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS*
902 (grey bars) and *coi1* lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount
903 of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error
904 bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown.
905 Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
906 (P<0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN*
907 *REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14*
908 *REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; UTR, untranslated
909 region; *GUS*, β -glucuronidase.

910 **Figure 10.** JA-induced polyamine biosynthesis depends on COI1-mediated JA signaling. (A)
911 Relative expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing
912 *pCaMV35S::GUS* (grey bars) and *coi1* lines (pink bars) were treated for 24 h with 50 μ M of JA
913 or an equal amount of ethanol. For control samples, cDNA of three biological replicates was
914 pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes
915 relative to the mean of three independent mock-treated control lines. Error bars denote standard
916 error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance
917 was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different
918 letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine
919 analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *coi1*
920 lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars
921 represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote
922 standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical
923 significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated
924 by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

925 **Figure 11.** Genome editing of a G-box decreases constitutive *C5-SD2* expression. (A) Schematic
926 representation of *C5-SD2* with location of the CRISPR-Cas9 cleavage site and G-box mutant
927 sequences. Dark grey boxes represent exons, solid lines represent introns, and light grey boxes
928 represent UTRs. The Cas9 cleavage site for the guide RNA targeting the G-box is indicated with
929 an arrowhead. Sequences of three independent G-box mutant (*g*) lines are shown. G-box
930 sequence is indicated in green font, the Cas9(VQR) PAM is marked in purple, inserted bases are

931 shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between
932 parentheses. Bases that make up an alternative G-box are green underlined. (B) Relative
933 expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR.
934 Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *g* lines (purple bars) were
935 treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of
936 three biological replicates was pooled per independent line and treatment. Bars represent mean
937 \log_2 -transformed fold changes relative to the mean of three independent mock-treated control
938 lines. Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are
939 shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
940 (P<0.05; indicated by different letters). (C) Tobacco BY-2 protoplasts were transfected with a
941 *pC5-SD2(g^{#1}; 1406 bp)::fLUC*, *pC5-SD2(g^{#2}; 1406 bp)::fLUC* or *pC5-SD2(g^{#3}; 1406 bp)::fLUC*
942 reporter construct and effector constructs overexpressing *MYC2* and *GAME9*. A
943 *pCaMV35S::rLUC* construct was co-transfected for normalization of fLUC activity. Bars
944 represent mean fold changes relative to the mean of protoplasts transfected with a *pC5-SD2(WT;*
945 *1406 bp)::fLUC* reporter construct and a *pCaMV35S::GUS* control construct. Dashed lines
946 represent the 2-fold cut off for promoter transactivation. Error bars denote standard error (n = 8).
947 Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
948 (P<0.05; indicated by different letters). (D) Relative accumulation of α -tomatine and
949 dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS*
950 (grey bars) and *g* lines (purple bars) were treated for 24 h with 50 μ M of JA or an equal amount
951 of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error
952 bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown.
953 Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis
954 (P<0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN*
955 *REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14*
956 *REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; UTR, untranslated
957 region; PAM, protospacer adjacent motif; *GUS*, β -glucuronidase; BY-2, Bright Yellow-2; *pC5-*
958 *SD2*, promoter of *C5-SD2*.

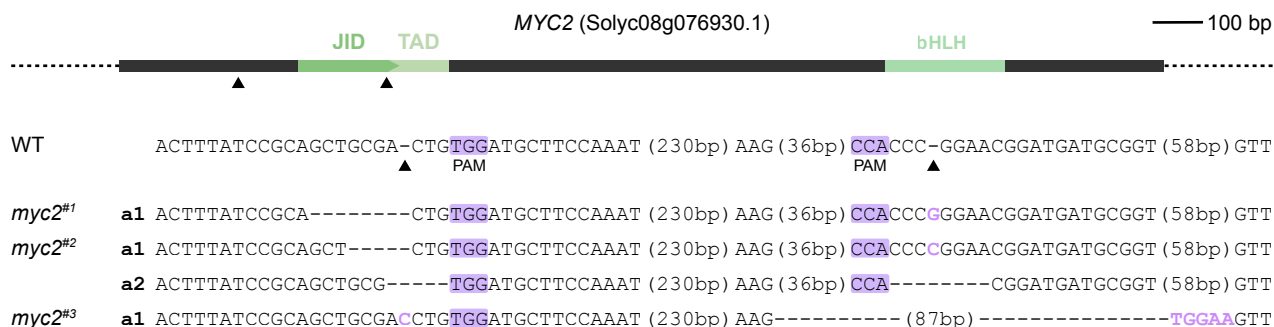
959 TABLES

Table 1. Genes Differentially Expressed Between Mock-Treated Wild-Type and *MYC2-RNAi* Tomato Seedlings That Belong to bHLH Clade IIIe or Are Responsible for SGA Biosynthesis

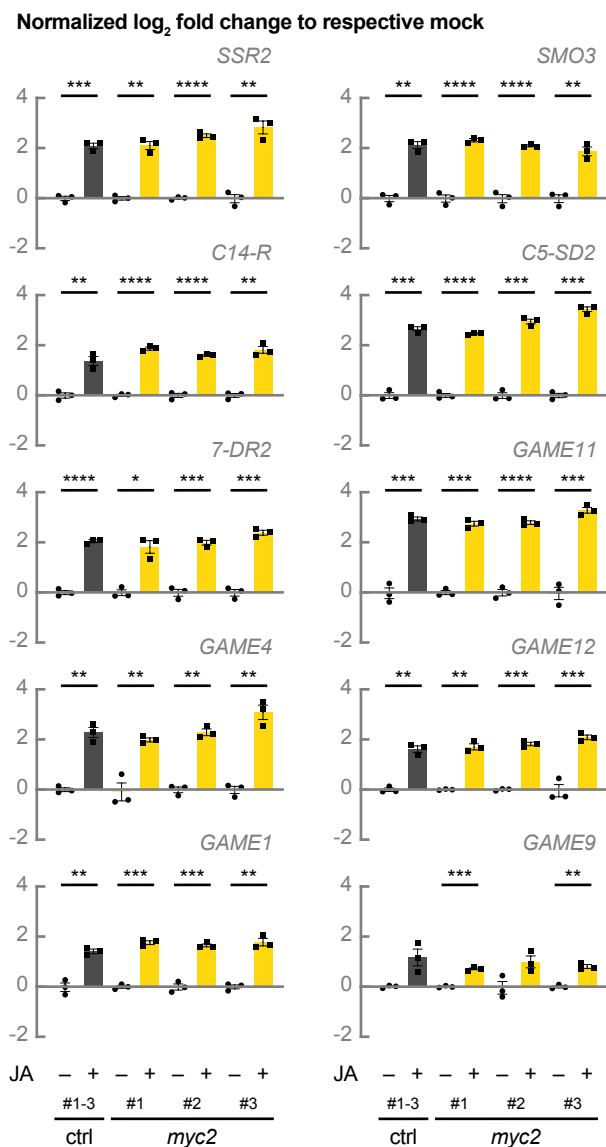
SolycID	Gene Name	Log ₂ Fold Change ^a	Adjusted P Value
bHLH Clade IIIe Transcription Factors:			
Solyc08g076930.1.1	<i>MYC2</i>	-1.33	1.28×10^{-31}
Solyc08g005050.2.1	<i>MYC1</i>	-0.45	3.82×10^{-2}
Cholesterol and Phytosterol Biosynthesis Pathway:			
Solyc04g070980.2.1	<i>CAS</i>	-0.53	1.08×10^{-9}
Solyc02g081730.2.1	<i>3bHSD2</i>	-0.78	1.36×10^{-7}
Solyc12g098640.1.1	<i>CPI</i>	-0.44	3.75×10^{-3}
Solyc01g008110.2.1	<i>CYP51</i>	-0.34	6.91×10^{-6}
Solyc09g009040.2.1	<i>C14-R</i>	-0.55	4.35×10^{-5}
Solyc06g082980.2.1	<i>8,7-SI</i>	-0.98	2.50×10^{-24}
Cholesterol Biosynthesis Pathway:			
Solyc02g069490.2.1	<i>SSR2</i>	-1.07	1.79×10^{-8}
Solyc01g091320.2.1	<i>SMO3</i>	-1.20	2.13×10^{-19}
Solyc06g005750.2.1	<i>SMO4</i>	-0.85	1.09×10^{-15}
Solyc02g086180.2.1	<i>C5-SD2</i>	-1.60	6.10×10^{-66}
Solyc06g074090.2.1	<i>7-DR2</i>	-0.97	3.09×10^{-9}
SGA Biosynthesis Pathway:			
Solyc07g043420.2.1	<i>GAME11</i>	-1.39	3.93×10^{-139}
Solyc07g043460.2.1	<i>GAME6</i>	-1.52	5.27×10^{-69}
Solyc12g006460.1.1	<i>GAME4</i>	-0.70	4.63×10^{-12}
Solyc12g006470.1.1	<i>GAME12</i>	-1.15	2.25×10^{-106}
Solyc01g073640.2.1	<i>GAME25</i>	-0.87	3.67×10^{-46}
Solyc07g043490.1.1	<i>GAME1</i>	-1.13	8.10×10^{-21}
Solyc07g043480.1.1	<i>GAME17</i>	-0.89	5.49×10^{-14}
Solyc07g043500.1.1	<i>GAME18</i>	-1.31	8.40×10^{-20}
RNA-seq data was previously published in (Du et al., 2017).			
^a Mean expression ratios calculated from three biologically independent experiments.			

960

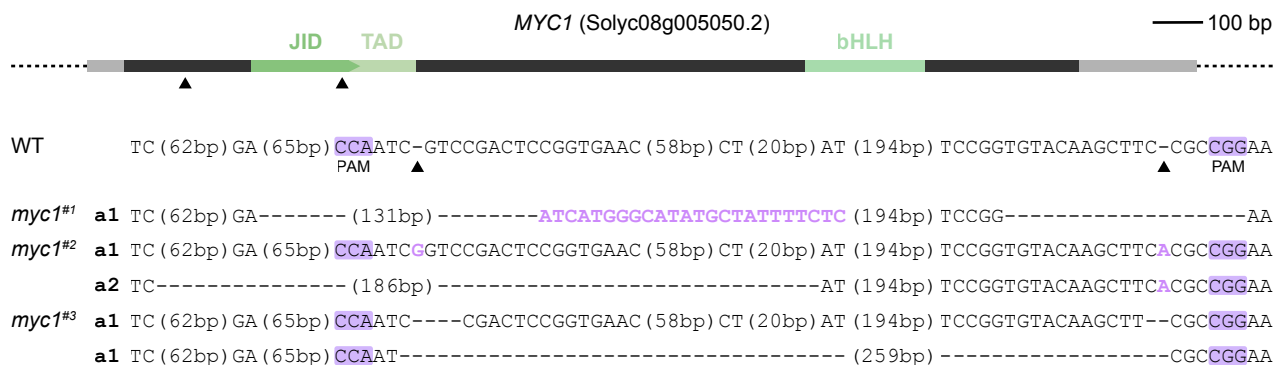
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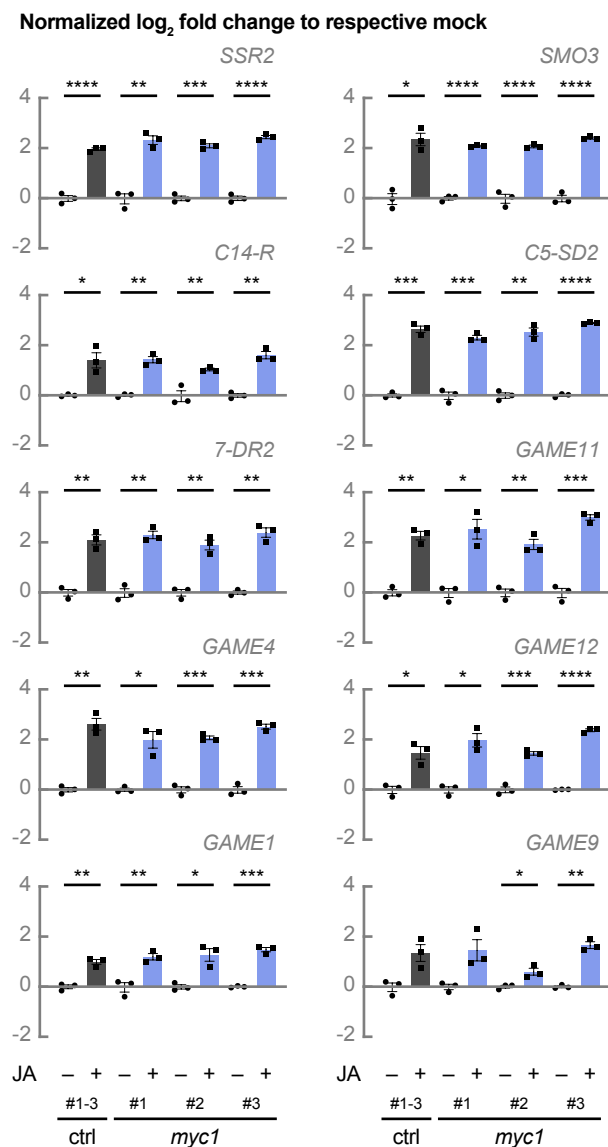
Supplemental Figure 1. Schematic representation of *MYC2* with location of the CRISPR-Cas9 cleavage sites and *myc2* mutant sequences. The dark grey box represents the exon. Green boxes represent encoded protein domains. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. Allele sequences of three independent *myc2* lines are shown. PAMs are marked in purple, inserted bases are shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between parentheses. Abbreviations: JID, JAZ interaction domain; TAD, transactivation domain; WT, wild-type; PAM, protospacer adjacent motif.



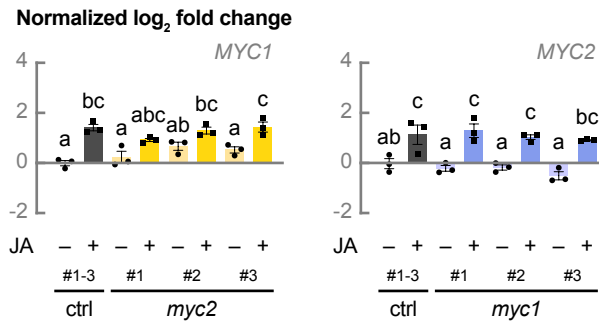
Supplemental Figure 2. Unaffected JA inducibility of SGA biosynthesis gene expression in *myc2* lines. Alternative representation of relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* shown in Figure 1. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of the respective mock-treated line. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -glucuronidase.



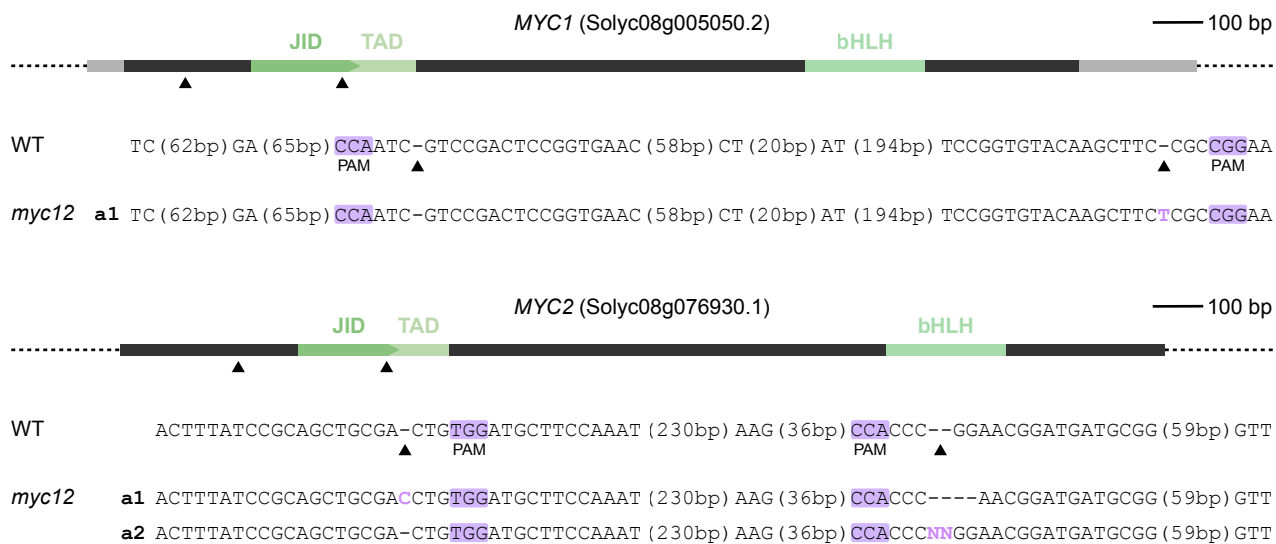
Supplemental Figure 3. Schematic representation of *MYC1* with location of the CRISPR-Cas9 cleavage sites and *myc1* mutant sequences. The dark grey box represents the exon and light grey boxes represent UTRs. Green boxes represent encoded protein domains. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. Allele sequences of three independent *myc1* lines are shown. PAMs are marked in purple, inserted bases are shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between parentheses. Abbreviations: JID, JAZ interaction domain; TAD, transactivation domain; WT, wild-type; PAM, protospacer adjacent motif; UTR, untranslated region.



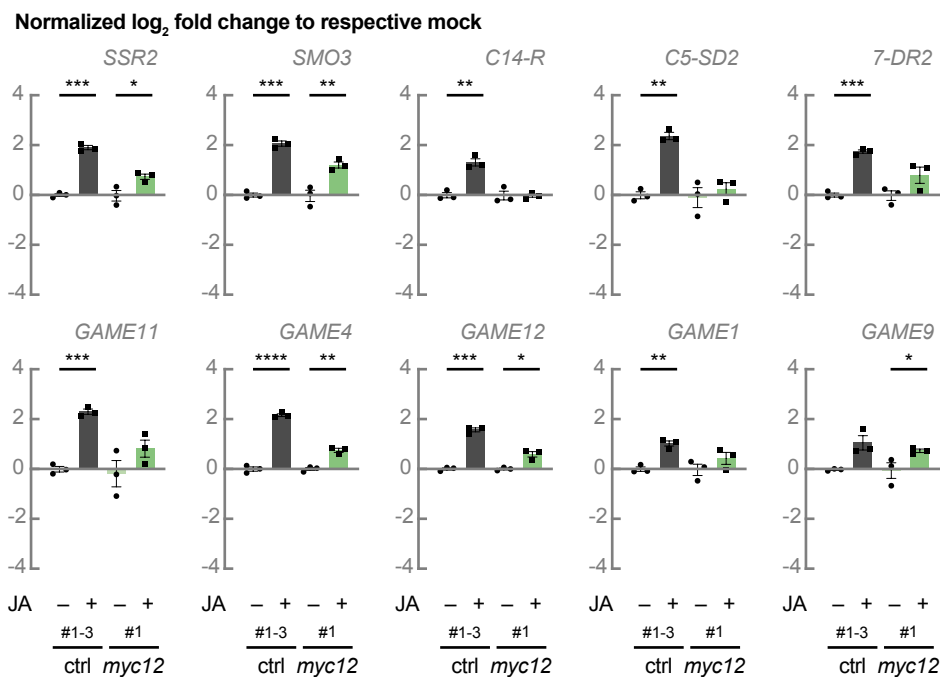
Supplemental Figure 4. Unaffected JA inducibility of SGA biosynthesis gene expression in *myc1* lines. Alternative representation of relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* shown in Figure 5. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of the respective mock-treated line. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -glucuronidase.



Supplemental Figure 5. *myc2* and *myc1* lines do not exhibit upregulated expression of *MYC1* and *MYC2*, respectively. Relative expression of *MYC1* and *MYC2* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars), *myc2* (yellow bars), and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P < 0.05; indicated by different letters). Abbreviations: *GUS*, β -glucuronidase.



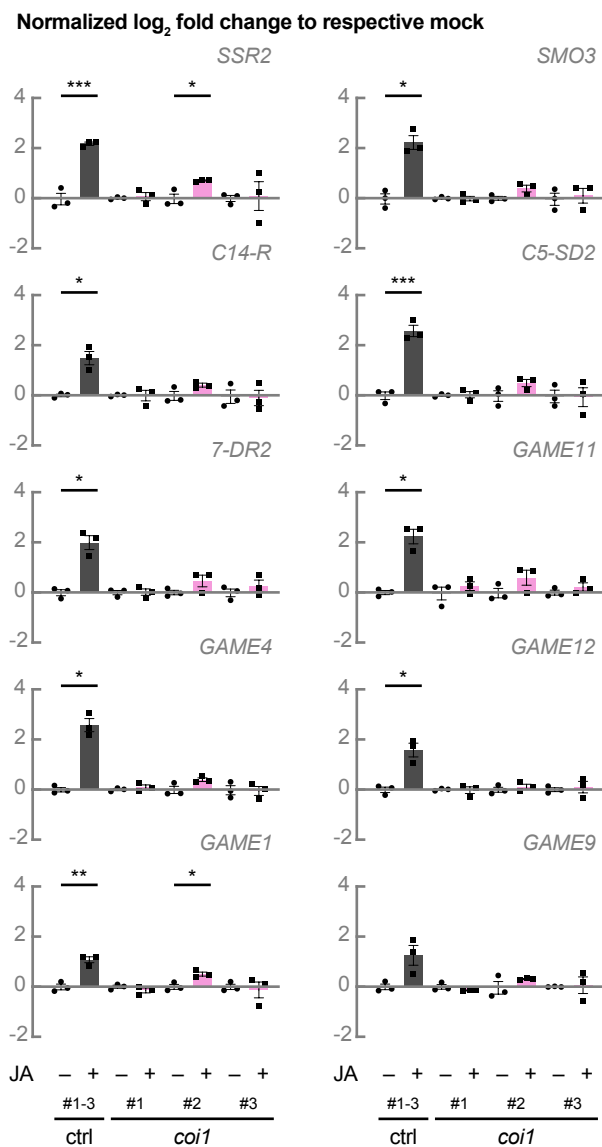
Supplemental Figure 6. Schematic representation of *MYC1* and *MYC2* with location of the CRISPR-Cas9 cleavage sites and *myc1 myc2* mutant sequences. Dark grey boxes represent exons and light grey boxes represent UTRs. Green boxes represent encoded protein domains. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. Allele sequences of one independent *myc1 myc2* (*myc12*) line are shown. PAMs are marked in purple, inserted bases are shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between parentheses. Abbreviations: JID, JAZ interaction domain; TAD, transactivation domain; WT, wild-type; PAM, protospacer adjacent motif; UTR, untranslated region.



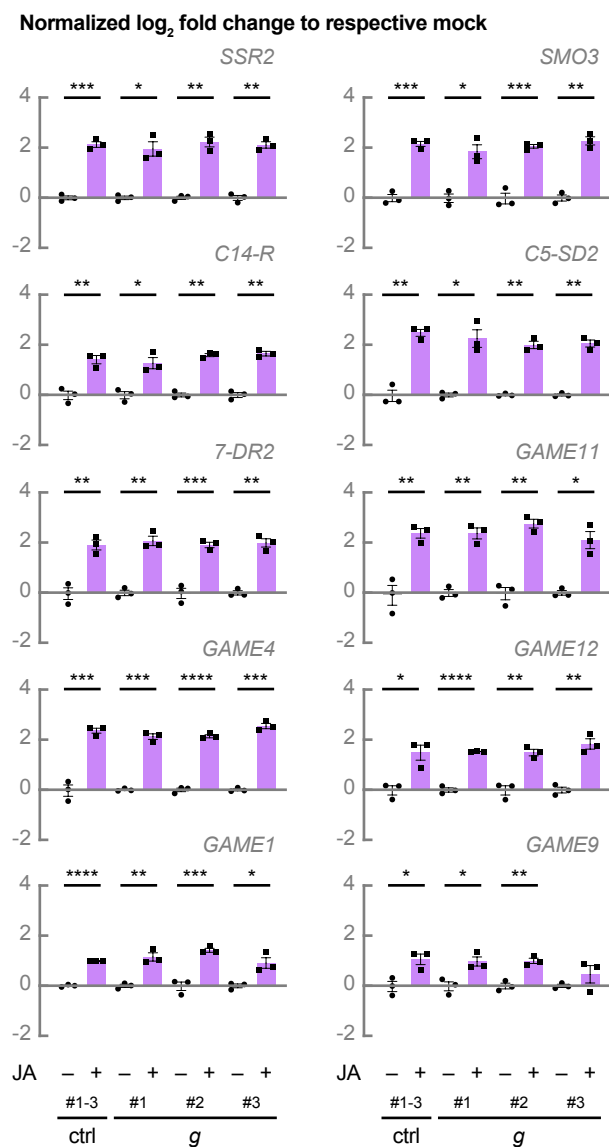
Supplemental Figure 7. Reduced or absent JA inducibility of SGA biosynthesis gene expression in a *myc1 myc2* line. Alternative representation of relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* shown in Figure 7. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2* (*myc12*) line (green bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of the respective mock-treated line. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Abbreviations: SSR2, STEROL SIDE CHAIN REDUCTASE 2; SMO3, C-4 STEROL METHYL OXIDASE 3; C14-R, STEROL C-14 REDUCTASE; 7-DR2, 7-DEHYDROCHOLESTEROL REDUCTASE 2; GUS, β-glucuronidase.



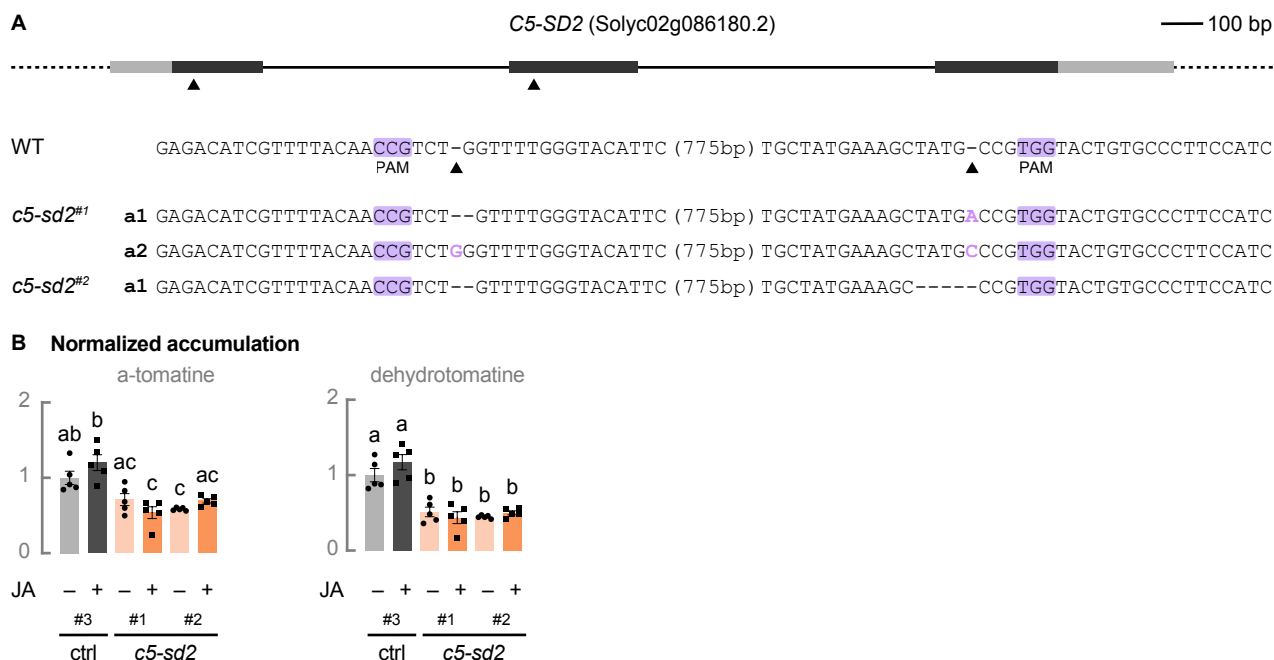
Supplemental Figure 8. Schematic representation of *COI1* with location of the CRISPR-Cas9 cleavage sites and *coi1* mutant sequences. Dark grey boxes represent exons, solid lines introns, and light grey boxes UTRs. Green boxes represent encoded protein domains. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. Allele sequences of three independent *coi1* lines are shown. PAMs are marked in purple, inserted bases are shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between parentheses. Abbreviations: LRR, leucine-rich repeat; WT, wild-type; PAM, protospacer adjacent motif; UTR, untranslated region.



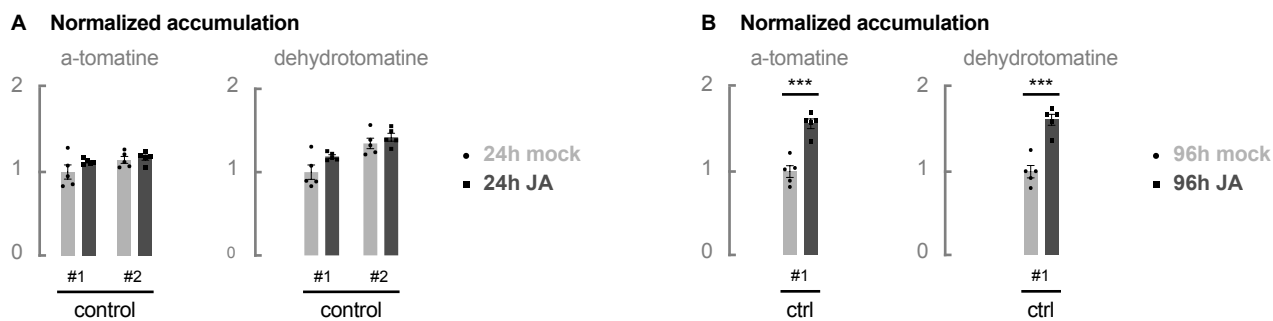
Supplemental Figure 9. SGA biosynthesis gene expression is no longer JA inducible in *coi1* lines. Alternative representation of relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* shown in Figure 9. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *coi1* lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of the respective mock-treated line. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -glucuronidase.



Supplemental Figure 10. JA inducibility of *C5-SD2* expression is not affected in G-box (*g*) mutant lines. Alternative representation of relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* shown in Figure 11. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *g* lines (purple bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of the respective mock-treated line. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -glucuronidase.



Supplemental Figure 11. Reduced SGA levels in *c5-sd2* lines. (A) Schematic representation of *C5-SD2* with location of the CRISPR-Cas9 cleavage sites and *c5-sd2* mutant sequences. Dark grey boxes represent exons, solid lines introns, and light grey boxes UTRs. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. Allele sequences of two independent *c5-sd2* lines are shown. PAMs are marked in purple, inserted bases are shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between parentheses. (B) Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *c5-sd2* lines (orange bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of the mock-treated control. Error bars denote standard error (n=5). Individual mock-treated (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: WT, wild-type; PAM, protospacer adjacent motif; UTR, untranslated region; *GUS*, β -glucuronidase.



Supplemental Figure 12. Limited SGA induction upon 96 h of JA treatment. Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* were treated for 24 h (A) or 96 h (B) with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of the leftmost mock-treated control. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Abbreviations: *GUS*, β -glucuronidase.


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AT5G46760 (AtMYC3)          351 SV--S-G-S-N-N-E-G-M-L-S-F--G---T---G---S---G---N---D---S-D-H-S-D-L-E-A-S-V-V-K-E---V-A-V-E---P-E-K-K-P---R-K-R
AT4G17880 (AtMYC4)          355 F-V--S---N-N-E-E-G-M-L-S-F-T-S---L-P-E---C---P---S-D-H-S-D-L-E-A-S-V-V-K-E---S-N-R-V-V-E---P-E-K-K-P---R-K-R
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bHLH

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AT5G46760 (AtMYC3)          403 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-S-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-S-Y-I-N-E-L-R---S-K-L---Q---A-E-S
AT4G17880 (AtMYC4)          404 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-S-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-S-Y-I-S-L-R---S-K-L---Q---A-E-S
AT5G46830 (AtMYC5)          331 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-C-V-I-N-E-L-R---S-K-L---H-N-E-S
CAN.G298.8
CAN.G1216.16
PGSC0003DMG400017535 (StJAMYC2) 430 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-A-Y-I-N-E-L-R---S-K-L---Q---A-E-S
PGSC0003DMG 400001161 (StJAMYC10) 503 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-A-Y-I-N-E-L-R---S-K-L---Q---A-E-S
Solyc08g005050.2 (SlMYC1)       413 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-A-Y-I-N-E-L-R---S-K-L---Q---A-E-S
Solyc08g076930.1.1 (SlMYC2)     445 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-A-Y-I-N-E-L-R---S-K-L---Q---A-E-S
NF_001312960.1 (NtMYC1a)        489 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-S-Y-I-N-E-L-R---S-K-L---Q---A-E-S
NF_001313001.1 (NtMYC1b)        487 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-S-Y-I-N-E-L-R---S-K-L---Q---A-E-S
NF_001312938.1 (NtMYC2a)        475 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-A-I-N-E-L-R---S-K-L---Q---A-E-S
NF_001311866.1 (NtMYC2b)        474 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-A-I-N-E-L-R---S-K-L---Q---A-E-S
Peaxil162Scf00045g00011         461 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-S-Y-I-N-E-L-R---S-K-L---Q---A-E-S
Peaxil162Scf00460g00011         488 G--R--K-P--A-N-G-R-E-E-P-L-N---H---V-E-A-E-R-Q-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A---I-S-Y-I-N-E-L-R---S-K-L---Q---A-E-S
AT4G37850 (AtbHLH25)           136 P-K-R-A-C-L-F-S---E-N-Q-S---N-A-Q-D-E---H---A-E-R-K-R-R-E-K-L-N-Q-R-F-Y-A-L-R-A-V-V-P-N-V-S-K-M-D-K-A-S-L-L-G-D-A-L-K-H-K-V-I-E-S-V-G-E-L-E---E---E
    
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bHLH

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AT1G32640 (AtMYC2)          507 R-L-Q---E---K-N-K---E-E-V---K-I-D-L-A---G---K-A-S-A-S---E---G---E-N-S---S-S-C---S-S---K-P-V-G-M---I---V-K-I-I---G-W-D
AT5G46760 (AtMYC3)          470 D-R-E---L---Q-K---K-L-G-S-K---E---N-N-G---K-G-C-G-S---A-K-E-R-K-S-S-N---Q-E---S-I-A---S-S-I---M---I-D-V-K-I-I---G-W-D
AT4G17880 (AtMYC4)          471 D-R-E---L---Q-K---Q-L-V-N-K-E---A---G-N-A-K-S---S-V---D-R-K---C-L-N---Q---S-S-V---V---E---M---D-V-K-I-I---G-W-D
AT5G46830 (AtMYC5)          398 K-H-A-I---E---E---F-N-E-L---K-B-L-A---G-Q---S---N---A-I---S---S-V-C-K-Y-E---K-A-S---E---E---K-I-V-K-I-I---E-S-D
CAN.G298.8
CAN.G1216.16
PGSC0003DMG400017535 (StJAMYC2) 497 N-I-E---L---L---K-S-Q-I-E-D-L-K---E-L-A---N---K-G-S---N-Y-F---P-S-P-P---Q-N---O-D-L---K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
PGSC0003DMG 400001161 (StJAMYC10) 570 D-R-E---L---K-S-Q-I-E-D-L-K---E-L-A---N---K-G-S---N-Y-S---S-S---P-P-S-N---O-D-L---K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
Solyc08g005050.2 (SlMYC1)       512 D-R-E---L---K-S-Q-I-E-D-L-K---E-L-T---N---K-G-S---N-Y-S---S-S---P-P---I-N---O-D-L---K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
Solyc08g076930.1.1 (SlMYC2)     568 D-R-E---L---K-S-Q-I-E-D-L-K---E-L-A---N---K-G-S---N-Y-S---S-S---P-P-P-P---P---O-D-L-K-M-S-S-H-T---G---S-K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
NF_001312960.1 (NtMYC1a)        556 D-R-E---L---K-S-Q-I-E-D-L-K---E-L-D---S---R-D---S---R-R-P---G-P---P-P---P---N---O-D-L-K-M-S-S-H-T---G---S-K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
NF_001313001.1 (NtMYC1b)        554 D-R-E---L---K-S-Q-I-E-D-L-K---E-L-V---S---R-D---S---R-R-P---G-P---P-P---S---N---H---K-M-S-S-H-T---G---S-K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
NF_001312938.1 (NtMYC2a)        542 D-R-E---L---N-O-I-E-S-L-N---E-L-A---N---K-G-S---N-Y-T---G-P---P-P---S---N---O-D-L---K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
NF_001311866.1 (NtMYC2b)        541 D-R-E---L---N-O-I-E-S-L-N---E-L-A---N---K-G-S---N-Y-T---G-P---P-P---P---N---O-D-L---K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
Peaxil162Scf00045g00011         528 E-E-E---L---K-S-Q-E-D-L-K-R---E-L-A---N---K-G-S---N-Y-S---G-P-S-P-P---N---O-D-L---K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
Peaxil162Scf00460g00011         555 D-R-E---L---K-S-Q-E-D-L-K-R---E-L-A---S---K-E-S---H-S---G-P---P-P---K---O-D-L-T-M-S-S-H-T---G---S-K-I---V-D-M---D-I-D-V-K-I-I---G-W-D
AT4G37850 (AtbHLH25)           207 Q-K-K---E---E---R---E-S-E---V---L-V---K-K-S---L-L-L-D---D-N---N---C-S-F-S---S-S---C-E-D-G-F-S-D---V---D---P-E-L-V-E---F---S-D
    
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AT1G32640 (AtMYC2)          557 --AMIRVSSR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
AT5G46760 (AtMYC3)          523 --VMIRVQCR--KD--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
AT4G17880 (AtMYC4)          520 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
AT5G46830 (AtMYC5)          444 --DAMVRE--SE--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
CAN.G298.8
CAN.G1216.16
PGSC0003DMG400017535 (StJAMYC2) 547 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
PGSC0003DMG 400001161 (StJAMYC10) 621 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
Solyc08g005050.2 (SlMYC1)       529 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
Solyc08g076930.1.1 (SlMYC2)     561 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
NF_001312960.1 (NtMYC1a)        620 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
NF_001313001.1 (NtMYC1b)        612 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
NF_001312938.1 (NtMYC2a)        610 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
NF_001311866.1 (NtMYC2b)        590 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
Peaxil162Scf00045g00011         589 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
Peaxil162Scf00460g00011         576 --AMIRVQCR--KN--HPAARLMA--LKE--LD--LDV--HNASVSVN---DI--MI--QQAATVVMGSRVYTB--Q--LR--I
AT4G37850 (AtbHLH25)           256 EDVLIILICEHQRE--H--LA--M--E--E--E--L--H--L--T--N--S--SV--N--FGPTLITLAKKESDFPM--H--HM--VVKVRS--
    
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AT1G32640 (AtMYC2)          616 S L S K I -----
AT5G46760 (AtMYC3)          583 A L L K R G E N ---Y
AT4G17880 (AtMYC4)          580 A L L E K Y G S P ---
AT5G46830 (AtMYC5)          506 ---L T S R - S -----
CAN.G298.8                   681 A L L T S R - T A E ---R
CAN.G1216.16                 607 A L L T S R - T A E ---R
PGSC0003DMG400017535 (StJAMYC2) 681 A L L T S R - T A E P L E S R
PGSC0003DMG 400001161 (StJAMYC10) 589 A L L T S R - T A E ---R
Solyc08g005050.2 (SlMYC1)    621 A L L T S R - T A E ---R
Solyc08g076930.1.1 (SlMYC2)  680 A L L T S R - T A E ---H
NF_001312960.1 (NtMYC1a)    672 A L L T S R - T A E ---R
NF_001313001.1 (NtMYC1b)    670 A L L T S R - T A E ---R
NF_001312938.1 (NtMYC2a)    650 S L L T S R - T A E ---R
NF_001311866.1 (NtMYC2b)    649 S L L T S R - T A E ---R
Peaxi162Scf00045g00011      636 A L L T S R - T A E ---R
Peaxi162Scf00460g00011      671 A L L T S R - T A E ---R
AT4G37850 (AtbHLH25)        323 A L L S N E F -----
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Supplemental Figure 13. Protein alignment generated by MUSCLE used for the phylogenetic tree in Figure 3A.

Supplemental Table 1. Oligonucleotides used in this study

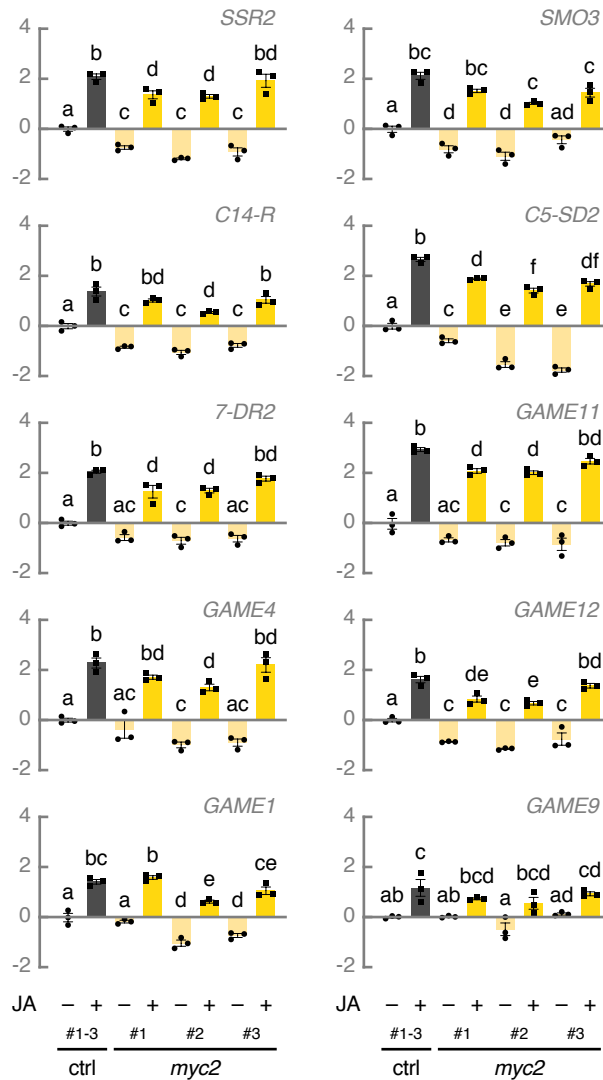
Oligonucleotide	Sequence (5'-3')	Orientation	Description	SolycID
Oligonucleotides for transient expression assay constructs:				
COMBI3834	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGACGGACTATAGATTATG	Forward	amplification of <i>MYC1</i>	Solyc08g005050.2
COMBI3835	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATCGCGATTCAGCAATTT	Reverse		
COMBI4483	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGGAAAATAACAACGGAGAGAG	Forward	amplification of <i>C5-SD2</i> promoter	Solyc02g086180.2
COMBI4484	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATAAATAAATAAATAAATTCTC	Reverse	from <i>g</i> lines	
Oligonucleotides for CRISPR-Cas9 constructs:				
COMBI6291	ATTGAGCTCACACACAACACGTG	Forward	G-box (in p <i>C5-SD2</i>) gRNA target site	Solyc02g086180.2
COMBI6292	AAACCACGTGTTGTGTGGTGTGAGCT	Reverse		
LAPAU3201	ATTGGTTCACCGGAGTCGGACGAT	Forward	<i>MYC1</i> gRNA target site 1	Solyc08g005050.2
LAPAU3202	AAACATCGTCCGACTCCGGTGAAC	Reverse		
LAPAU3203	ATTGTCCGGTGTACAAGCTTCCGC	Forward	<i>MYC1</i> gRNA target site 2	Solyc08g005050.2
LAPAU3204	AAACGCGGAAGCTGTACACCGGA	Reverse		
LAPAU3195	ATTGTTTATCCGCAGCTGCGACTG	Forward	<i>MYC2</i> gRNA target site 1	Solyc08g076930.1
LAPAU3196	AAACCAGTCGCAGCTGCGGATAAA	Reverse		
LAPAU3197	ATTGACCGCATCATCCGTTCCGGG	Forward	<i>MYC2</i> gRNA target site 2	Solyc08g076930.1
LAPAU3198	AAACCCGGAACGGATGATGCGGT	Reverse		
LAPAU3207	ATTGTGATAGAGACGCGGTATCGT	Forward	<i>COI1</i> gRNA target site 1	Solyc05g052620.2
LAPAU3208	AAACACGATACCGCTCTCTATCA	Reverse		
LAPAU3209	ATTGAAATTAACATAGCAGCTCT	Forward	<i>COI1</i> gRNA target site 2	Solyc05g052620.2
LAPAU3210	AAACAGAGCTGCTATGTTAATTT	Reverse		
COMBI6283	ATTGAGAATGTACCCAAAACCAGA	Forward	<i>C5-SD2</i> gRNA target site 1	Solyc02g086180.2
COMBI6284	AAACTCTGGTTTTGGGTACATTCT	Reverse		
COMBI6285	ATTGTTGCTATGAAAGCTATGCCG	Forward	<i>C5-SD2</i> gRNA target site 2	Solyc02g086180.2
COMBI6286	AAACCGGCATAGCTTTCATAGCAA	Reverse		
attB5_AtU6gRNA	GGGGACAACCTTTGTATACAAAAGTTGTACTTTTTTCTTCTTCTTCGTTTCATACAG	Forward	gRNA module with attB5 and attB4	–
attB4_AtU6gRNA	GGGGACAACCTTTGTATAGAAAAGTTGGGTGCTAGAAAAAAGCACCAGACTCGG	Reverse	flanking sites	
attB4r_AtU6gRNA	GGGGACAACCTTTTCTATACAAAAGTTGTACTTTTTTCTTCTTCTTCGTTTCATACAG	Forward	gRNA module with attB4r and attB3r	–
attB3r_AtU6gRNA	GGGGACAACCTTTATTATACAAAAGTTGTCTAGAAAAAAGCACCAGACTCGG	Reverse	flanking sites	
attB3_AtU6gRNA	GGGGACAACCTTTGTATAATAAAGTTGTACTTTTTTCTTCTTCTTCGTTTCATACAG	Forward	gRNA module with attB3 and attB2	–
attB2_AtU6gRNA	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGAAAAAAGCACCAGACTCGG	Reverse	flanking sites	
noBbsI_F	AGTCTTGCGACTGAGCCTTTCGTTTTATTTGATGCC	Forward	Eliminate <i>BbsI</i> site in pDONR221	–
noBbsI_R	CTCAGTCGCAAGACTGGGCCTTTCGTTTTATCTG	Reverse		
Oligonucleotides for the identification of CRISPR-Cas9 hairy root mutants:				
LAPAU3075	TCCCTCATCAGATCCACCTC	Forward	amplification of Cas9	–
LAPAU3076	CTGAAACCTGAGCCTTCTGG	Reverse		
COMBI6305	CCTCCATCTCCGACGGTATACTAGG	Forward	amplification of G-box (in p <i>C5-SD2</i>)	Solyc02g086180.2
COMBI6306	CTGCAACAAGTGGCTGGTTAGTTC	Reverse	gRNA target region	
LAPAU3205	CTACTCTCATTTCTCACCTAACAAACAAAATCT	Forward	amplification of <i>MYC1</i> gRNA target	Solyc08g005050.2
LAPAU3206	GCCTGGCCCGTTCACAT	Reverse	region	
LAPAU3199	TGCCACCATGAATTTGTGGA	Forward	amplification of <i>MYC2</i> gRNA target	Solyc08g076930.1
LAPAU3200	ATCGTCTGAAGCCGAACC	Reverse	region	

LAPAU3211	CTCTCTCCTCCATCTTCTTCAACTG	Forward	amplification of <i>CO11</i> gRNA target	Solyc05g052620.2
LAPAU3212	GCAGGAACGAGAAATATGCAGAAGAC	Reverse	region	
COMBI6287	GTGTTACCGCACTAGCCCCCTC	Forward	amplification of <i>C5-SD2</i> gRNA target	Solyc02g086180.2
COMBI6288	GGAGGTGGCCTACTGTCAATCAG	Reverse	region 1	
COMBI6289	CTGATTGACAGTAGGCCACCTCC	Forward	amplification of <i>C5-SD2</i> gRNA target	Solyc02g086180.2
COMBI6290	GGTTGCGTGTGCGATTAGCC	Reverse	region 2	
Oligonucleotides for gene expression analysis by qPCR:				
COMBI5428	CCTCCGTTGTGATGTAAGTGG	Forward	amplification of <i>CAC</i>	Solyc08g006960.2
COMBI5429	ATTGGTGGAAAGTAACATCATCG	Reverse		
COMBI5416	ATGGAGTTTTGAGTCTTCTGC	Forward	amplification of <i>TIP41</i>	Solyc10g049850.1
COMBI5417	GCTGCGTTTCTGGCTTAGG	Reverse		
COMBI5683	GGCCAAATGTCAAGGGTCACT	Forward	amplification of <i>SSR2</i>	Solyc02g069490.2
COMBI5684	ACCCGAACCCATTGATCA	Reverse		
COMBI7957	CCCACTTTTGGTTCCTTGTC	Forward	amplification of <i>SMO3</i>	Solyc01g091320.2
COMBI7958	GGAAATTCATACCCGCTGTG	Reverse		
COMBI7951	CGTTTGTCCACGTTGCTTGTC	Forward	amplification of <i>C14-R</i>	Solyc09g009040.2
COMBI7952	TTGGGTAGTGCAGTACAGTGAG	Reverse		
COMBI6281	TTCGTGGAAGCCTTATGGAC	Forward	amplification of <i>C5-SD2</i>	Solyc02g086180.2
COMBI6282	TGGCGGTATGTTGATGGTG	Reverse		
COMBI5669	TCTGCTTGGGCGTTTCTTC	Forward	amplification of <i>7-DR2</i>	Solyc06g074090.2
COMBI5670	CCAAACCACCCTGCCTTTTC	Reverse		
COMBI5756	TGTGCAGCCTATTCGCAATG	Forward	amplification of <i>GAME11</i>	Solyc07g043420.2
COMBI5757	TTTGTACACACGATGTGC	Reverse		
COMBI5673	ACCTGTTGCTCTTATGTCTGTC	Forward	amplification of <i>GAME4</i>	Solyc12g006460.1
COMBI5674	CCTCTTGTTCCTTTGGCTT	Reverse		
COMBI5677	GCGGAGGTTCTTATGTCTATG	Forward	amplification of <i>GAME12</i>	Solyc12g006470.1
COMBI5678	GCTTCAATAAGACGAGGCTCAC	Reverse		
COMBI5671	TTGCCGGATGTTCCATGATCG	Forward	amplification of <i>GAME1</i>	Solyc07g043490.1
COMBI5672	CTAATGAAGAAACAGCGTCTGG	Reverse		
COMBI6976	ACCAAGCCGCTTACAAGATTCGG	Forward	amplification of <i>GAME9</i>	Solyc01g090340.2
COMBI6977	TAGGTACGTCCGAGCCAATCAG	Reverse		
COMBI6817	GATTTACCAATCGCGAGACG	Forward	amplification of <i>JAZ1</i>	Solyc12g009220.1
COMBI6818	TTGAGCACCTAATCCCAACC	Reverse		
COMBI6946	AGACGCCGATTCCAACGCTTATC	Forward	amplification of <i>ODC</i>	Solyc04g082030.1
COMBI6947	TCCGAGTTGAGCTGCTGTTTCG	Reverse		
COMBI7491	AAGGAGGCCGTTGTAGAACCTG	Forward	amplification of <i>MYC1</i>	Solyc08g005050.2
COMBI7492	TCCCTTCCATTGGCTGGTTTCC	Reverse		
COMBI5852	TAATGGAAGTGGGCTTCCTG	Forward	amplification of <i>MYC2</i>	Solyc08g076930.1
COMBI5853	GCTGCCAATTTCTCAGTTCC	Reverse		

A *MYC2* (Solyc08g076930.1) — 100 bp



B Normalized log₂ fold change



C Normalized accumulation

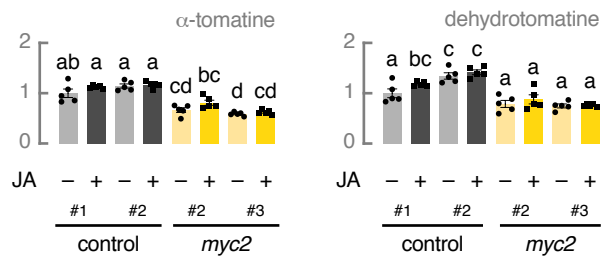


Figure 1. MYC2 coordinates constitutive SGA biosynthesis in tomato. (A) Schematic representation of *MYC2* with location of the CRISPR-Cas9 cleavage sites. The dark grey box represents the exon. Cas9 cleavage sites for two guide RNAs are indicated with arrowheads. (B) Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean \log_2 -transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (C) Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -glucuronidase.

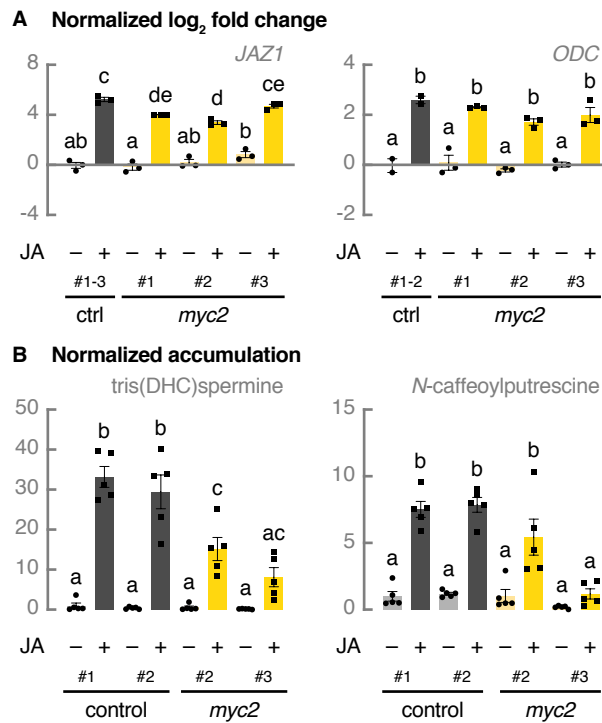


Figure 2. MYC2 helps ensure JA-induced polyamine production in tomato. (A) Relative expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc2* lines (yellow bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

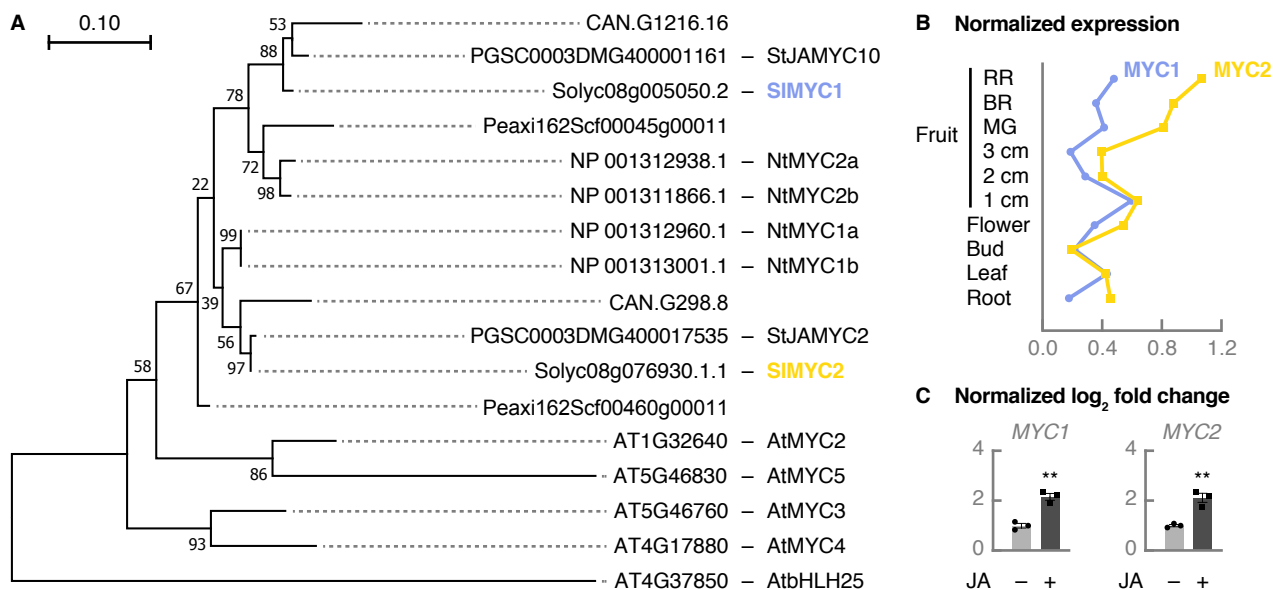


Figure 3. Tomato has two clade IIIe bHLH family members. (A) Phylogenetic analysis of *Arabidopsis thaliana* and Solanaceae bHLH clade IIIe members using MUSCLE and the Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved amino acid sequences from *Capsicum annuum* (CAN.G1216.16, CAN.G298.8), *Solanum tuberosum* (PGSC0003DMG400001161, PGSC0003DMG400017535), *S. lycopersicum* (Solyc08g005050.2, Solyc08g076930.1.1), *Petunia axillaris* (Peaxi162Scf00045g00011, Peaxi162Scf00460g00011), *Nicotiana tabacum* (NP_001312938.1, NP_001311866.1, NP_001312960.1, NP_001313001.1), and *A. thaliana* (AT1G32640, AT5G46830, AT5G46760, AT4G17880). The *A. thaliana* bHLH clade IVa member bHLH25 (AT4G37850) was used to root the tree. Numbers shown are bootstrap values in percentages (based on 1,000 replicates). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). (B) Normalized *MYC1* and *MYC2* expression profiles in different organs and developmental stages (cultivar Heinz 1706). Expression data were obtained from TomExpress (Zouine et al., 2017). (C) Relative expression of *MYC1* and *MYC2* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. The cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by unpaired Student's *t*-tests (**, P<0.01). Abbreviations: 1 cm, 1 cm immature green fruit; 2 cm, 2 cm immature green fruit; 3 cm, 3 cm immature green fruit; MG, mature green fruit; BR, breaker fruit; RR, red ripe (breaker + 10 days) fruit.

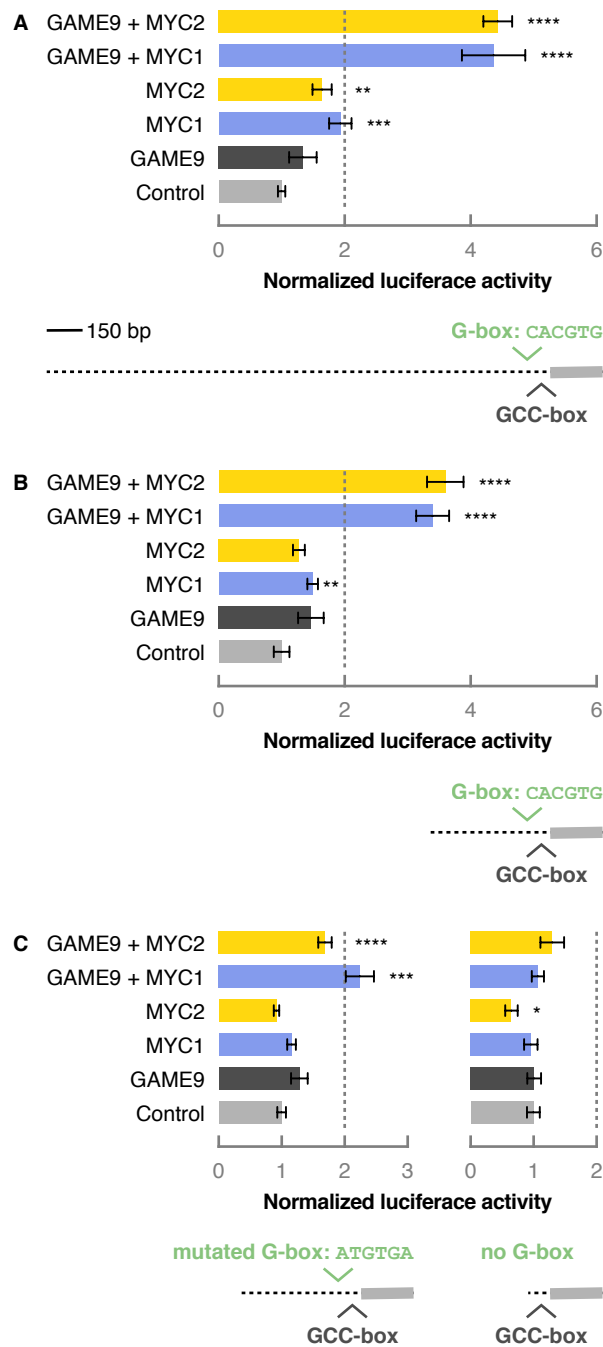
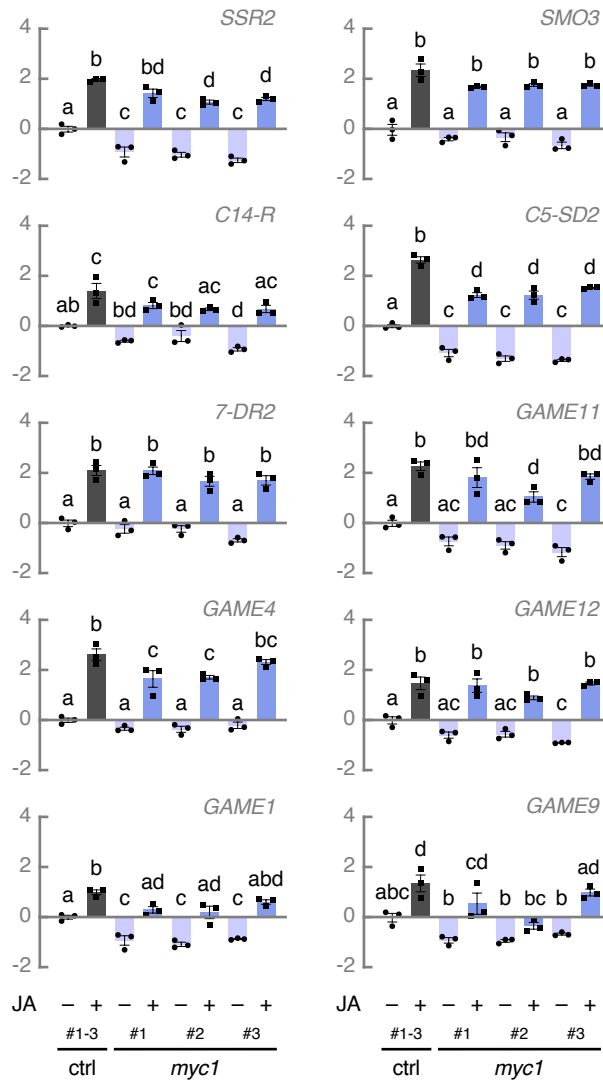


Figure 4. MYC1 and MYC2 transactivate the promoter of *C5-SD2* together with GAME9. (A-C) Tobacco BY-2 protoplasts were transfected with a *pC5-SD2(1549 bp)::fLUC* (A), *pC5-SD2(333 bp)::fLUC* (B), *pC5-SD2(333 bp with mutated G-box)::fLUC* or *pC5-SD2(207 bp without G-box)::fLUC* (C) reporter construct and effector constructs overexpressing *MYC1*, *MYC2*, *GAME9* or a combination thereof. A *pCaMV35S::rLUC* construct was co-transfected for normalization of fLUC activity. Bars represent mean fold changes relative to the mean of protoplasts transfected with a *pCaMV35S::GUS* control construct (grey bar). Dashed lines represent the 2-fold cut off for promoter transactivation. Error bars denote standard error (n = 8). Statistical significance was determined by unpaired Student's *t*-tests (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). Abbreviations: BY-2, Bright Yellow-2; *pC5-SD2*, promoter of *C5-SD2*; *GUS*, β -glucuronidase.

A *MYC1* (Solyc08g005050.2) — 100 bp



B Normalized log₂ fold change



C Normalized accumulation

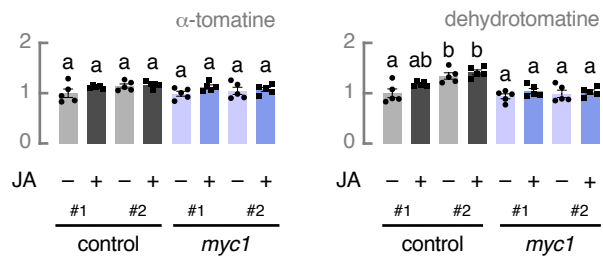


Figure 5. MYC1 regulates constitutive expression of SGA biosynthesis genes in tomato. (A) Schematic representation of *MYC1* with location of the CRISPR-Cas9 cleavage sites. The dark grey box represents the exon and light grey boxes represent the UTRs. Cas9 cleavage sites for two guide RNAs are indicated with arrowheads. (B) Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean \log_2 -transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P < 0.05; indicated by different letters). (C) Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P < 0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; UTR, untranslated region; *GUS*, β -glucuronidase.

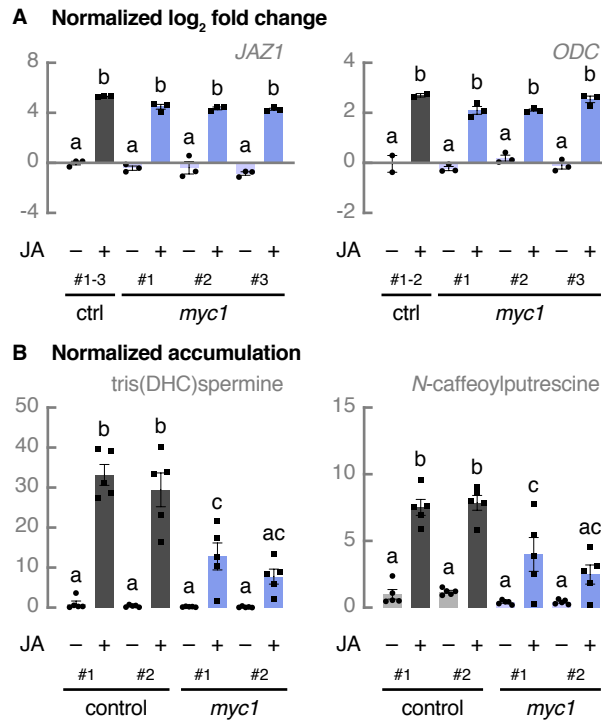


Figure 6. MYC1 helps ensure JA-induced polyamine biosynthesis in tomato. (A) Relative expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *myc1* lines (blue bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

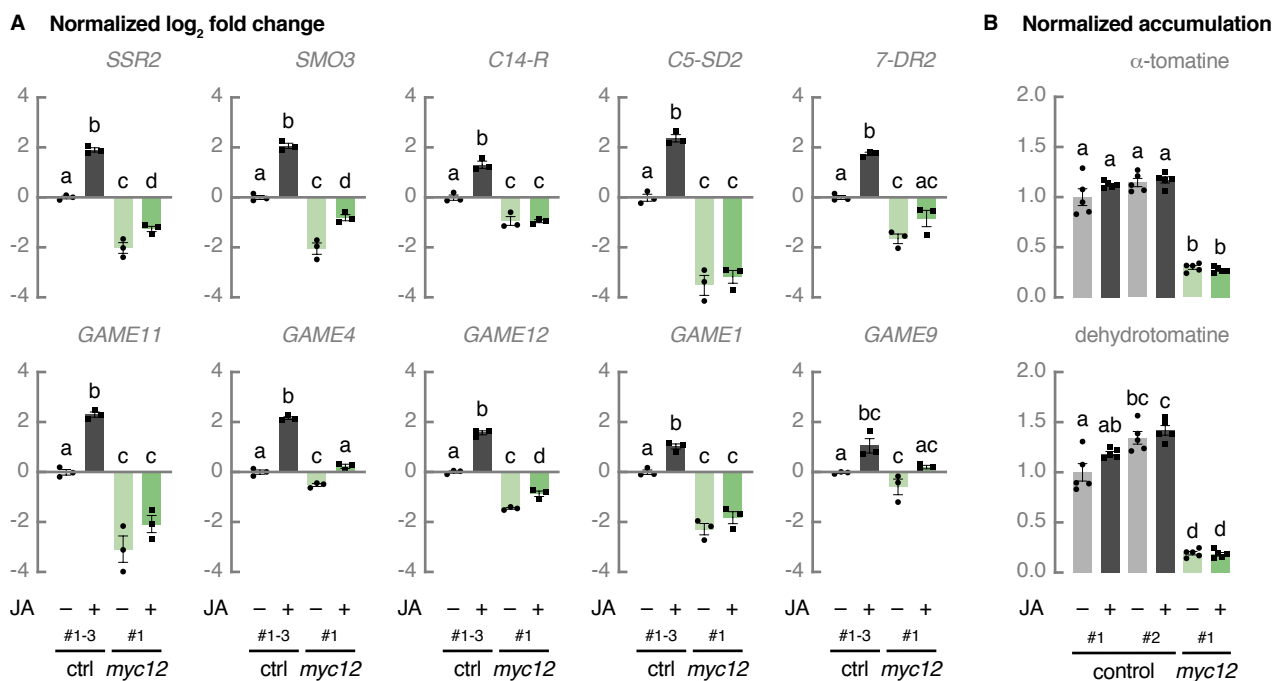


Figure 7. MYC1 and MYC2 redundantly regulate constitutive SGA biosynthesis in tomato. (A) Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2* (*myc12*) line (green bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (B) Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2* (*myc12*) line (green bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n = 5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P < 0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; *GUS*, β -glucuronidase.

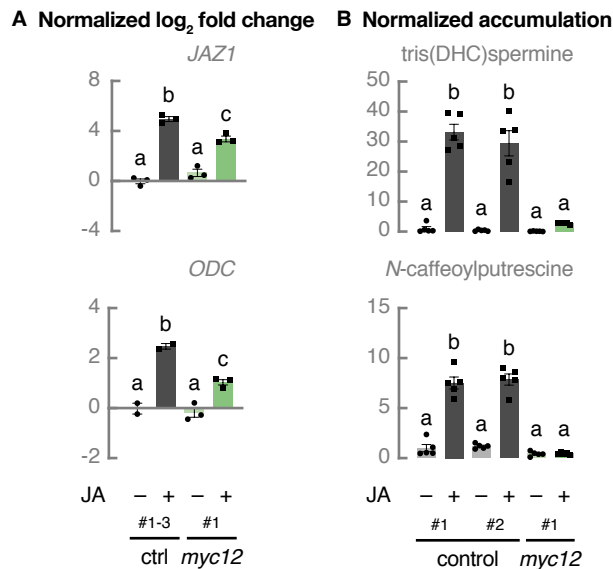


Figure 8. MYC1 and MYC2 redundantly regulate JA-induced polyamine accumulation in tomato. (A) Relative expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2* (*myc12*) line (green bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and a *myc1 myc2* (*myc12*) line (green bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.

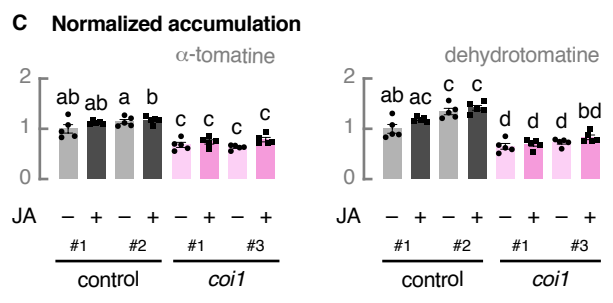
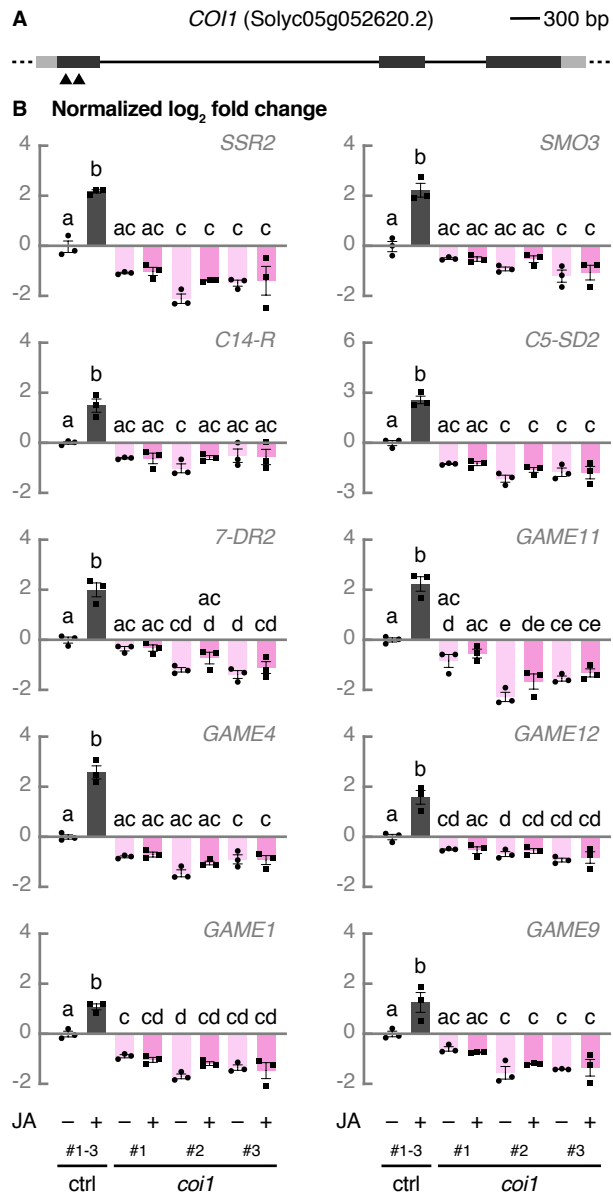


Figure 9. Constitutive SGA biosynthesis partially depends on COI1-mediated JA signaling. (A) Schematic representation of *COI1* with location of the CRISPR-Cas9 cleavage sites. Dark grey boxes represent exons, solid lines represent introns, and light grey boxes represent UTRs. Cas9 cleavage sites for two guide RNAs are indicated with arrowheads. (B) Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *coil* lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean \log_2 -transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters). (C) Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *coil* lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; UTR, untranslated region; *GUS*, β -glucuronidase.

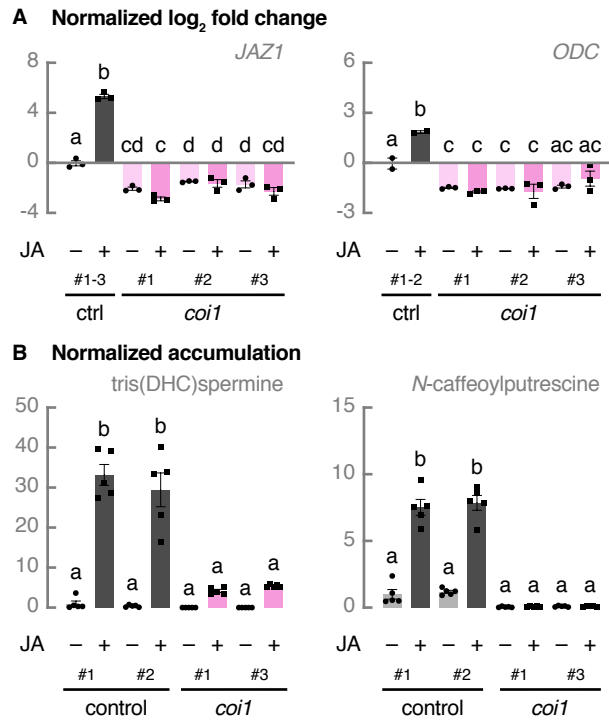
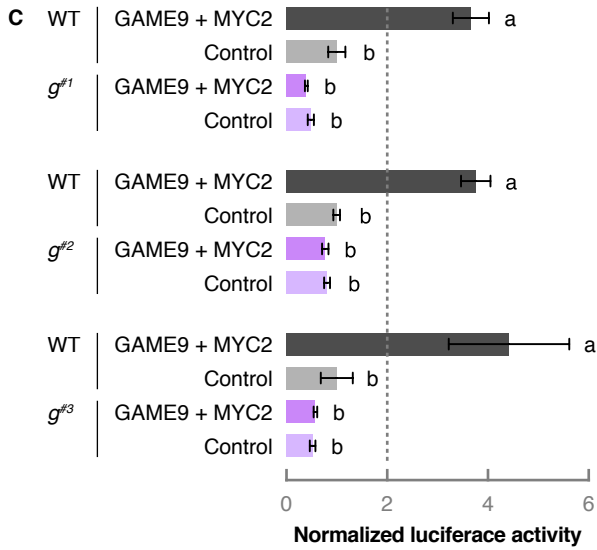
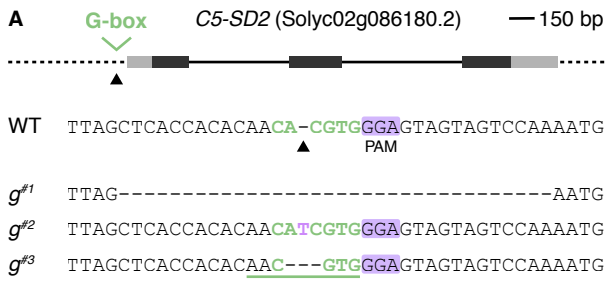
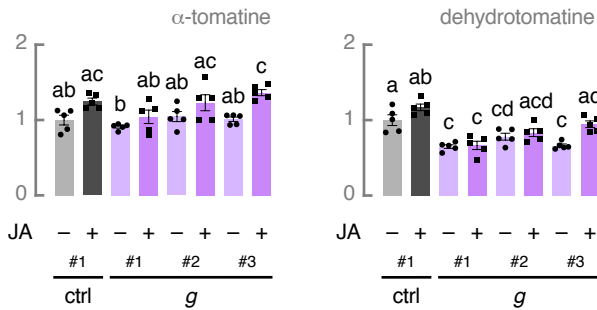


Figure 10. JA-induced polyamine biosynthesis depends on COI1-mediated JA signaling. (A) Relative expression of *JAZ1* and *ODC* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *coi1* lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean log₂-transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n = 3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (B) Relative accumulation of tris(dihydrocaffeoyl)spermine and *N*-caffeoylputrescine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *coi1* lines (pink bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: DHC, dihydrocaffeoyl; *GUS*, β -glucuronidase.



D Normalized accumulation



B Normalized log₂ fold change

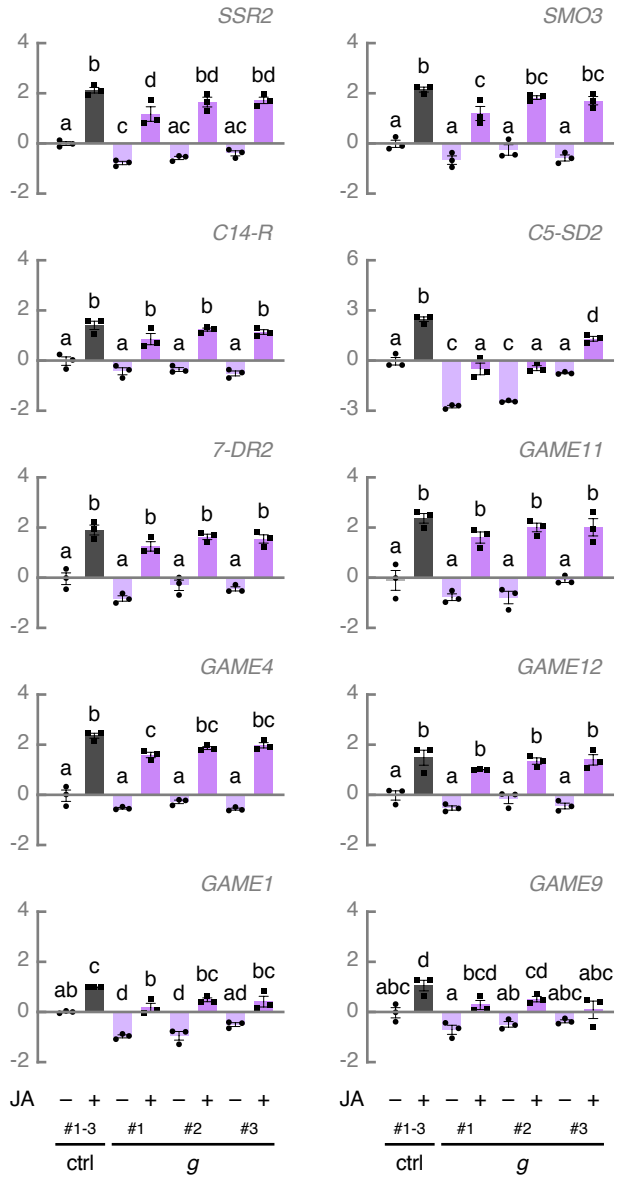


Figure 11. Genome editing of a G-box decreases constitutive *C5-SD2* expression. (A) Schematic representation of *C5-SD2* with location of the CRISPR-Cas9 cleavage site and G-box mutant sequences. Dark grey boxes represent exons, solid lines represent introns, and light grey boxes represent UTRs. The Cas9 cleavage site for the guide RNA targeting the G-box is indicated with an arrowhead. Sequences of three independent G-box mutant (*g*) lines are shown. G-box sequence is indicated in green font, the Cas9(VQR) PAM is marked in purple, inserted bases are shown in purple, deleted bases are replaced by a dash, and sequence gap length is shown between parentheses. Bases that make up an alternative G-box are green underlined. (B) Relative expression of cholesterologenesis genes, SGA biosynthesis genes, and *GAME9* analyzed by qPCR. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *g* lines (purple bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. For control samples, cDNA of three biological replicates was pooled per independent line and treatment. Bars represent mean \log_2 -transformed fold changes relative to the mean of three independent mock-treated control lines. Error bars denote standard error (n=3). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (C) Tobacco BY-2 protoplasts were transfected with a *pC5-SD2(g^{#1}; 1406 bp)::fLUC*, *pC5-SD2(g^{#2}; 1406 bp)::fLUC* or *pC5-SD2(g^{#3}; 1406 bp)::fLUC* reporter construct and effector constructs overexpressing *MYC2* and *GAME9*. A *pCaMV35S::rLUC* construct was co-transfected for normalization of fLUC activity. Bars represent mean fold changes relative to the mean of protoplasts transfected with a *pC5-SD2(WT; 1406 bp)::fLUC* reporter construct and a *pCaMV35S::GUS* control construct. Dashed lines represent the 2-fold cut off for promoter transactivation. Error bars denote standard error (n = 8). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). (D) Relative accumulation of α -tomatine and dehydrotomatine analyzed by LC-MS. Control hairy root lines expressing *pCaMV35S::GUS* (grey bars) and *g* lines (purple bars) were treated for 24 h with 50 μ M of JA or an equal amount of ethanol. Bars represent mean fold changes relative to the mean of mock-treated control^{#1}. Error bars denote standard error (n=5). Individual mock- (●) and JA-treated (■) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis (P<0.05; indicated by different letters). Abbreviations: *SSR2*, *STEROL SIDE CHAIN REDUCTASE 2*; *SMO3*, *C-4 STEROL METHYL OXIDASE 3*; *C14-R*, *STEROL C-14 REDUCTASE*; *7-DR2*, *7-DEHYDROCHOLESTEROL REDUCTASE 2*; UTR, untranslated region; PAM, protospacer adjacent motif; *GUS*, β -glucuronidase; BY-2, Bright Yellow-2; *pC5-SD2*, promoter of *C5-SD2*.

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