# Steroidal glycoalkaloids contribute to anthracnose resistance in *Solanum lycopersicum*

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## Highlight

This work describes an important role for steroidal glycoalkaloids (SGAs), abundant secondary metabolites in tomato fruit, in defense against anthracnose, a widespread fungal disease that impacts diverse crop species.


## Abstract

Anthracnose is a widespread plant disease caused by various species of the fungal pathogen *Colletotrichum*. In solanaceous plants such as tomato (*Solanum lycoperiscum*), *Colletotrichum* infections exhibit a quiescent, asymptomatic state in developing fruit, followed by a transition to necrotrophic infections in ripe fruit. Through analysis of fruit tissue extracts of 95L368, a tomato breeding line that yields fruit with enhanced anthracnose resistance, we identified a role for steroidal glycoalkaloids (SGAs) in anthracnose resistance. The SGA α-tomatine and several of its derivatives accumulated at higher levels, in comparison to fruit of the susceptible tomato cultivar US28, 95L368 fruit extracts with fungistatic activity against *Colletotrichum*. Correspondingly, ripe and unripe 95L368 fruit displayed enhanced expression of glycoalkaloid metabolic enzyme (GAME) genes, which encode key enzymes in SGA biosynthesis. Metabolomics analysis incorporating recombinant inbred lines (RILs) generated from 95L368 and US28 yielded strong positive correlations between anthracnose resistance and accumulation of α-tomatine and several derivatives. Lastly, transient silencing of expression of the GAME genes *GAME31* and *GAME5* in anthracnose-susceptible tomato fruit yielded enhancements to anthracnose resistance. Together, our data support a role for SGAs in anthracnose defense in tomato, with a distinct SGA metabolomic profile conferring resistance to virulent *Colletotrichum* infections in ripe fruit.



## Keywords

anthracnose, Colletotrichum, GAME2, GAME31, GAME4, GAME5, metabolomics, Solanum lycopersicum, steroidal glycoalkaloids, tomato


## Abbreviations

| | |
|---|---|
| GAME: | glycoalkaloid metabolic enzyme |
| MALDI-TOF: | matrix-assisted laser desorption/ionization time-of-flight |
| MS: | mass spectrometry |
| SGA: | steroidal glycoalkalkoid |
| UHPLC: | ultra-high performance liquid chromatography |

## Introduction

Anthracnose is a global plant disease caused by *Colletotrichum* spp., hemibiotrophic fungal pathogens with a wide and diverse host range, including a variety of commercially important monocot and dicot crops such as tomato, maize, strawberry, and soybean (Dias et al., 2019; Jacobs et al., 2019; Prochno et al., 2016; Stommel and Haynes, 1998). Plant pathogens in both the field and post-harvest, *Colletotrichum* spp. encompass approximately 190 species in eleven species complexes, exhibiting multiple infection strategies in various types of plant tissue, including foliar, fruit, and root tissue, depending on the pathosystem (Baroncelli et al., 2017; Boufleur et al., 2021; Jayawardena et al., 2016). In solanaceous crops such as pepper and tomato, anthracnose is caused primarily by the species *C. coccodes*, *C. gloeosporioides*, *C. dematium*, and *C. acutatum*, and is characterized by the formation of necrotrophic lesions on fruit (Chapin et al., 2006; Harp et al., 2014; Pardo-De la Hoz et al., 2016). *Colletotrichum*

infections of tomato fruit exhibit a transition from quiescence to virulence during fruit ripening, yielding anthracnose symptoms on ripe fruit. After overwintering in soil, sclerotia form conidia capable of germinating on immature fruit, resulting in an asymptomatic, quiescent state characterized by the formation of appressoria that are restricted to the cuticle. As infected fruit ripens, *Colletotrichum* forms hyphae that penetrate the pericarp, and produces circular depressions that progress into necrotrophic lesions on mature, ripe fruit (Alkan et al., 2015; Peres et al., 2005; Sanogo et al., 2003). In the field, traditional strategies to mitigate anthracnose infection, such as fungicide application, provide incomplete protection, thus the development of genetic tools to improve anthracnose resistance is of particular interest (Byrne et al., 1997; Wyenandt et al., 2009). Anthracnose resistance has been identified in fruits of unadapted tomato germplasm, and attempts to breed this trait into elite materials convey a polygenic, additive mode of inheritance, as well as difficulty in transfer to commercial cultivars with an intensity equal to that observed in the resistant germplasm (Stommel, 2001; Stommel and Haynes, 1998). Therefore, more targeted approaches to develop enhanced anthracnose resistance in commercial cultivars are desirable.

Steroidal glycoalkaloids (SGAs) are secondary metabolites, derived from the mevalonate biosynthetic pathway and characterized by a nitrogenous aglycone backbone and variable glycone sidechains at the C-3 position, that accumulate to high levels in solanaceous plants such as tomato, eggplant, and potato (Cárdenas et al., 2015; You and Kan, 2021; Zhao et al., 2021). Found in various plant tissues, including leaves, fruit, and tubers, some SGAs are generally classified as antinutritional compounds due to the observed toxicity in humans, when ingested at high levels, but SGAs have also been described for their potential health benefits, including anti-inflammatory, anticarcinogenic, and antioxidative properties (Friedman, 2006, 2013; Wang et al., 2018). For example, in vitro and mouse model studies have reported activation of cancer cell apoptosis and reduction of tumor cell growth following treatments of α-tomatine, the most abundant SGA in tomato leaves and green fruit (Chao et al., 2012; Kim et al., 2015). Furthermore, numerous studies have characterized α-tomatine in responses to diverse sources of biotic stress in *Solanaceae* (Hoagland, 2009; Sandrock and VanEtten, 1998). Antifungal activity of α-tomatine was observed in vitro and in tomatoes infected with *Fusarium oxysporum*, a vascular fungal pathogen and the causal agent of fusarium wilt (Ito et al., 2007; Pareja-Jaime et al., 2008), and α-tomatine was shown to restrict in vitro growth of *Phytophthora infestans*, the oomycete pathogen that causes late blight in potato (Dahlin et al., 2017; Sandrock and VanEtten, 1998). Notably, a role for α-tomatine in defense against *Colletotrichum spp.* has been described, with α-tomatine treatment observed to restrict the growth of both *C. coccodes* and *C. acutatum*, and a transgenic tomato line with attenuated levels of α-tomatine displaying enhanced susceptibility to *C. coccodes* (Itkin et al., 2011; Sandrock and VanEtten, 1998). Accordingly, SGAs, including α-tomatine, are of particular interest in the development of genetic tools to imbue enhanced levels of defense against anthracnose and other biotic diseases of tomato.

While α-tomatine accumulates to high levels in immature fruit, levels of α-tomatine and certain derivative SGAs decline together during ripening, ultimately giving way to the derivative esculeosides, end products of the SGA pathway that accumulate tFig. o high levels in ripe fruit. GLYCOALKALOID METABOLIC ENZYME (GAME) genes encode enzymes that catalyze reactions in the SGA pathway (Fig. S1) (Akiyama et al., 2019; Cárdenas et al., 2019; Lee et al., 2019; Nakayasu et al., 2020; Sonawane et al., 2018). The UDP-glycosyltransferase GAME2 catalyzes the synthesis of α-tomatine, as well as the closely related dehydrotomatine, representing a branch point for the synthesis of downstream SGA derivatives (Iijima et al., 2013; Itkin et al., 2013; Kozukue et al., 2004). As tomato fruit ripens, these compounds are converted to derivative SGAs via additional GAME proteins with hydroxylation, acetyltransferase, and glycosylation activities. For example, *GAME31* encodes a 2-oxoglutarate-dependent dioxygenase (2-ODD) that synthesizes hydroxytomatine and hydroxy-dehydrotomatine from α-tomatine and dehydrotomatine, respectively (Nakayasu et al., 2020; Szymański et al., 2020). Additional, as-yet-undiscovered GAME proteins catalyze the acetoxylation of these substrates, respectively forming the derivative acetoxytomatine and acetoxy-dehydrotomatine, and the subsequent hydroxylation reactions that result in the formation of acetoxy-hydroxytomatine and acetoxy-hydroxy-dehydrotomatine, accordingly (Cárdenas et al., 2015; Zhao

113 et al., 2021). The uridine diphosphate (UDP) glycosyltransferase GAME5 synthesizes the formation of
114 esculeoside A and dehydro-esculeoside A from acetoxy-hydroxytomatine and acetoxy-hydroxy-
115 dehydrotomatine, respectively (Iijima et al., 2009; Nakayasu et al., 2020; Szymański et al., 2020). Although
116 the ripening process in tomato is generally characterized by a decline in α-tomatine levels and coincident
117 accumulation of the end-product esculeosides, comparisons of commercial tomato cultivars, wild tomato
118 accessions, and derivative introgression lines have illustrated a diversity of SGA metabolomic profiles in
119 ripe red fruit, with some lines exhibiting high levels of α-tomatine in both green and red fruit (Dzakovich et
120 al., 2021; Iijima et al., 2013; Szymański et al., 2020; Zhu et al., 2018). While a growing body of evidence
121 supports a role for α-tomatine in pathogen defense, little is known of the contributions of derivative SGAs,
122 therefore germplasm of variant SGA metabolomic profiles can be particularly useful in further research to
123 elucidate the role of SGAs in disease resistance in tomato.
124 Here our work incorporates an anthracnose-susceptible tomato cultivar (US28) and a small-fruited,
125 commercially unadapated and anthracnose-resistant accession (95L368), as well as a population of twenty-
126 eight selected recombinant inbred lines (RILs), in a study of the relationship between anthracnose defense
127 and the SGA metabolome. Red fruit tissue extracts of the anthracnose-resistant 95L368 exhibited
128 fungistatic activity against *Colletotrichum* and were found to be enriched in certain SGAs. Correspondingly,
129 gene expression analysis conveyed modulated transcription of GAME genes in 95L368 in both green and
130 red fruit. US28, 95L368, and the descendant RILs displayed variable anthracnose resistance, and
131 metabolomics analysis of this population yielded a distribution of SGA profiles in red fruit. Further analysis
132 of the SGA metabolic profiles identified positive correlations between anthracnose resistance and the α-
133 tomatine and dehydrotomatine derivatives hydroxytomatine, acetoxytomatine, and dehydro-
134 acetoxytomatine. Lastly, Virus-Induced Gene Silencing (VIGS) was employed to evaluate the roles of
135 *GAME31* and *GAME5* in anthracnose resistance, wherein silencing of these GAME genes conferred
136 enhanced levels of anthracnose resistance in a susceptible tomato line. Together, these data provide
137 meaningful insights into the contribution of SGAs to anthracnose defense in tomato, highlighting new targets
138 for the development of crop plants with enhanced biotic disease resistance.
139
140 **Materials and Methods**
141
142 **Plant material and *Colletotrichum* inoculations**
143
144 The F2 population derived from a cross between the parent lines *Solanum lycopersicum* US28 and
145 95L368 (formerly denoted 115-4) was generated as described (Stommel, 2001). Twenty-eight recombinant
146 inbred lines (RILs) were selected from a larger RIL population developed via single seed descent from the
147 F2 generation, with each RIL carried to the F6 or F7 generation. Seedlings for US28, 95L368 and the
148 population of 28 RILs were germinated in greenhouse conditions transferred to the field, and arranged into
149 four planting blocks, in Keyport fine loam soil, at USDA-ARS BARC-WEST, Beltsville MD, USA Fruit were
150 harvested at the young, mature green, breaker, and mature red stages, and stored, either whole or diced,
151 at -80 $^{o}$C for subsequent experiments. For *Colletotrichum* infection assays, mature red fruit from each line
152 (mean n = 22 fruit per block per line) were harvested and transferred to a greenhouse. *Colletotrichum* isolate
153 C9, previously isolated from tomato in Beltsville and tentatively identified as *C. nigrum*, was maintained and
154 prepared as previously described (Stommel and Haynes, 1998). Suspensions were diluted to a final
155 concentration of 5 x $10^6$ spores $mL^{-1}$ for inoculation. Fruit exocarp was wounded via 27-gauge needle,
156 inoculated with a 10 µL droplet of fungal suspension, and incubated in the greenhouse, with lesion diameter
157 (lesion size $\geq$ 1 mm) recorded at 7 dpi, and where noted, converted to "severity" via normalization to the
158 within-block maximum lesion size.
159
160 ***Colletotrichum* growth inhibition assays**
161

Approximately 300 mg red ripe fruit tissue from 95L368 samples stored at -80 °C was ground with liquid nitrogen in a mortar and pestle, left to thaw, then centrifuged at 13,000 rpm for one minute, after which the supernatant was collected. Supernatant was vortexed and centrifuged with equal parts sterile water and 100% MeOH, and samples of whole supernatant, MeOH fraction, water fraction, and MeOH (control) were spread on V8 agar plates. Spores of *Colletotrichum* C9 were harvested from V8 agar plates and suspended in chilled, sterile water, at a final concentration of 5 x $10^3$ µL. Aliquots of 10 µL of fungal suspension were added to the PDA plates and incubated at 25 °C for five days for observation of mycelial growth. For the TLC bioassay, 300 mg samples of lyophilized ripe fruit tissue were homogenized in liquid nitrogen, transferred to 10 mL 100% MeOH, and shaken at 250 rpm for 30 minutes. After centrifugation at 5,000 rpm for 15 minutes, 4 mL supernatant was added to 6 mL water, then passed through pre-conditioned (6 mL 100% MeOH followed by 6 mL water) C18 Sep-Pak Cartridges (Waters Corporation, Milford MA, USA) by gravity. After washing with 6 mL 100% MeOH, the samples were eluted with 15 mL 80% MeOH, dried via rotary evaporator, and resuspended in 3 mL 100% MeOH. Aliquots of 10 µL were spotted onto TLC plates and developed at room temperature. After drying the plates in a fume hood for 1 hour, the plates were photographed under UV light, then sprayed with a suspension of *Colletotrichum* C9 (3 x $10^6$ spores $mL^{-1}$) in sterile water and stored in closed petri dishes for 4-5 days before observation of mycelial growth. For the growth inhibition assay incorporating US28, 95L368, and the 4 select RILs, non-treated V8 agar plates were inoculated with *Colletotrichum* as described, and at 7 dpi, 80 µL of MeOH extractions were spotted along the plate perimeters, after which images were generated at 3 and 4 days post-treatment (dpt).

**SGA Metabolomics**

For MALDI-TOF MS, fruit tissue methanol extractions separated by C18 chromatography were prepared as previously described. Mass spectra were acquired in positive ion detection mode on a Bruker-Daltonics Microflex spectrometer (Bruker-Daltonics, Billerica MA, USA) For each sample, 2 µL of eluate was mixed with matrix (2,5-dihydrobenzoic acid) in 10 µL acetonitrile and added to 10 µL water, after which 0.5 µL spots of the eluate-matrix mix were added to the stainless-steel target surface and crystallized at room temperature. Each sample was measured in triplicate, with 3,000 laser pulses (λ = 337.1 nm) at 200 ns each, and with the following voltage settings: ion source 1 19.0 kV; ion source 14.0 kV; lens 9.2 kV; and reflector 20.0 kV. Data was processed via the supplied Bruker Daltonics software, and accurate masses were verified via SIS Exact Mass Calculator (https://www.sisweb.com/referenc/tools/exactmass.htm).

For UHPLC-HRMS full scanning, fruit tissue methanol extractions separated by C18 chromatography were prepared as previously described. The UHPLC-HRAM MS system consisted of an LTQ Orbitrap XL MS with an Agilent 1290 UHPLC (Agilent Technologies, Santa Clara CA, USA). Separation was conducted on a Hypersil Gold AQ RP-C18 UHPLC column (Thermo Fisher Scientific, Waltham MA, USA) with an UltraShield pre-column filter (Analytical Scientific Instruments, Richmond CA, USA). Gradient elution (Solution A, 0.1% v/v formic acid in water; Solution B, 0.1% formic acid in acetonitrile) was conducted at a flow rate of 0.3 $mL \cdot min^{-1}$. The linear gradient was as follows: increase from 2% to 10% Solution B (v/v) at 10 minutes; increase to 40% Solution B at 25 minutes; increase to 75% Solution B at 30 minutes; increase to 90% Solution B at 50 minutes; and hold at 90% Solution B to 60 minutes. The re-equilibration time for the initial gradient was 5 minutes. Column compartment temperature was set at 50 °C. Data were acquired under positive ionization mode and negative ionization mode with mass range of $m/z$ = 100-2,000 with a resolution of 30,000, separately. The optimized conditions for positive ionization mode were set as follows: sheath gas 80 AU; aux and sweep gas 15 AU; spray voltage 4.5 kV; capillary temperature 325 °C; capillary voltage 40 V; tube lens voltage 150 V; FTMS AGC target 5 x $10^5$; FT-MS/MS AGC target 2 x $10^5$; isolation width 1.5 atomic mass units (amu); and max ion injection time 200 ms. For negative ionization mode, the parameters were set as follows: spray voltage 4 kV; capillary voltage -50 V; and tube lens voltage -120 V. The most intense ion was selected for the data-dependent scan to observe their MS2 to MS4 product ions, respectively, with a normalization collision energy at 35%.

211 For targeted analysis of SGAs via UHPLC-HRMS, samples of 100 mg lyophilized red fruit tissue
212 were ground in a bead homogenizer with 1/4”-diameter ceramic spheres (MP Biomedicals, LLC, Irvine CA,
213 USA) and vortexed in 5 mL 100% MeOH for 10 seconds. Samples were shaken for 30 minutes at 250 rpm,
214 then centrifuged for 15 minutes at 5,000 rpm, at room temperature. For each sample, approximately 4 mL
215 supernatant was passed through 0.45 µM PTFE syringe filters, then stored at -20 ºC. Injection volumes of
216 1 µL were loaded onto a Q-Exactive mass spectrometer with a Thermo Fisher Vanquish UHPLC system.
217 Separation was conducted on an Agilent Poroshell HPH-C18 column. Gradient elution (Solution A, 0.1%
218 v/v formic acid in water; Solution B, 0.1% v/v formic acid in acetonitrile) was conducted with a flow rate of
219 0.3 mL $\cdot min^{-1}$, a sample compartment temperature of 5 ºC, and a column temperature of 40 ºC. Mass
220 spectra were generated via electrospray ionization with 4 kV spray voltage, an ion transfer tube temperature
221 of 320 ºC, and a scan range of m/z 120-1,800 Da. Ion chromatograms were constructed for the protonated
222 molecular ions of each analyte via high resolution ion extraction with a mass width of 0.01 amu, and the
223 SGA contents (µg g dry weight $(DW)^{-1}$) for all analytes were semi-quantitated as $\alpha$-tomatine equivalent from
224 an external calibration curve of $\alpha$-tomatine standard (Sigma-Aldrich, St. Louis MO, USA). For statistical
225 analyses, the SGA content values were $log_{10}$-transformed via the following formula: $log_{10}$-SGA = $log_{10}$ [(x
226 µg $gDW^{-1}$)+1)].
227
228 **Gene expression analysis**
229
230 Fresh-frozen or lyophilized samples of approximately 100 mg fruit tissue were ground in liquid
231 nitrogen in a mortar and pestle, or via bead homogenizer with 1/4”-diameter ceramic spheres (MP
232 Biomedicals, LLC, Irvine CA, USA). RNA was purified via RNEasy Mini Kit (Qiagen, Hilden DE), per
233 manufacturer’s instructions, with elutions in 30 µL RNase-free water. cDNA synthesis was conducted via
234 ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich MA, USA), using
235 manufacturer’s instructions for reverse transcription of total RNA. RNA masses and purities were analyzed
236 via a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington DE, USA). cDNA samples were diluted
237 100-fold for qRT-PCR. Primers for qRT-PCR were designed from target mRNA sequences, spanning
238 introns where possible, and synthesized by Integrated DNA Technologies, Inc. (Coralville IA, USA); see
239 Table S3 for a list of all primer sequences. Primers for GAME-VIGS qRT-PCR validation were designed to
240 yield amplicons without overlap to the VIGS target amplicons (see below). Primer efficiencies, specificities,
241 and optimal annealing temperatures were analyzed via CFX Maestro Software Version 2.2 (Bio-Rad). qRT-
242 PCR was carried out via an iProof SYBR Green Supermix kit (Bio-Rad Laboratories, Hercules CA, USA)
243 per manufacturer’s instructions. Two technical replicates were included for each sample. For each target
244 relative expression (RE) was calculated via the $2^{-ddCt}$ method, with the lowest-expression sample
245 designated as the calibrator.
246
247 **Virus-Induced Gene Silencing (VIGS) cloning and GAME-VIGS Agroinfiltration**
248
249 RNA and cDNA were isolated from fresh-frozen 95L368 fruit tissue samples as described above.
250 Primers designed to amplify a 406-bp region of *GAME31* coding sequence (CDS), as well as a 478-bp
251 region of *GAME5* CDS, were synthesized by Integrated DNA Technologies and are listed in Table S3.
252 Amplification of target amplicons with the addition of 5’ XbaI and 3’ BamHI restriction enzyme digestion
253 sites was carried out on a Bio-Rad T100 thermocycler (Bio-Rad Laboratories, Hercules CA, USA) via Bio-
254 Rad iProof High Fidelity DNA Polymerase using manufacturer’s instructions. Amplicons were separated via
255 gel electrophoresis on a Bio-Rad PowerPac Basic system, analyzed via an Azure c150 Gel Imaging System
256 (Azure Biosystems, Inc., Dublin CA, USA), and purified via Zymoclean Gel DNA Recovery kit (Zymo
257 Research, Irvine CA, USA) using manufacturer’s instructions. The bipartite VIGS system consisted of the
258 vectors pYL192 (TRV1) and pYL156 (TRV2). Double digestion of pYL156 and target amplicons was
259 conducted via Thermo Fisher FastDigest BamHI and XbaI using manufacturer’s instructions. Ligations

260 utilized New England Biolabs T4 ligase and were conducted at 16 °C overnight, followed by enzyme
261 deactivation at 65 °C for 10 minutes. Aliquots of 2 µL of each reaction were added to 50 µL high efficiency
262 New England Biolabs 5-alpha Competent *E. coli*, placed on ice for 30 minutes, incubated in a 42 °C water
263 bath for 40 seconds, then returned to ice for 2 minutes. After recovery in 1 mL LB for 1 hour, cells were
264 washed in 100 µL LB, plated on LB agar supplemented with 50 µg/mL kanamycin, and incubated at 38 °C
265 overnight. Positive clones were identified via PCR as previously described, using MangoMix Taq DNA
266 polymerase (Meridian Bioscience Inc., Cincinnati OH, USA) and manufacturer's instructions.
267 Putatively recombinant plasmid was isolated from overnight cultures using the Zymo Research
268 ZymoPURE Plasmid Miniprep Kit, per manufacturer's instructions. Positive recombinant vector was verified
269 via Sanger sequencing incorporating the NOS-R primer (Table S3) and performed by Psomagen (Rockville
270 MD, USA). Aliquots of 1 µL of TRV1 and recombinant TRV2 were electroporated into 50 µL tubes of
271 *Agrobacterium tumifasciens* strain GV3101 cells (Intact Genomics, St. Louis MO, USA) using a Bio-Rad
272 Gene Pulser II and Pulse Controller electroporation system set to 2.2 kV. Transformants were grown on LB
273 plates supplemented with 50 µg $mL^{-1}$ kanamycin and 25 µg $mL^{-1}$ spectinomycin and verified via PCR; see
274 Table S3 for primers. For agroinfiltration, 5 mL overnight cultures in LB supplemented with 50 µg $mL^{-1}$
275 kanamycin were transferred to 20 mL cultures and shaken at 225 rpm for 3 hours or until an approximate
276 $OD_{600}$ of 1.0 was reached, then pelleted and resuspended in 20 mL infiltration medium (200 µL 1M $MgCl_2$
277 + 200 µL 1M MES pH 5.6 + 20 µl 200 mM acetosyringone + 19.58 mL sterile water). After gentle shaking
278 for 3 hours, 1:3 TRV1:TRV2 mixed suspensions were prepared. Micro Tom tomato plants grown in
279 greenhouse conditions and presenting immature green fruit were agroinoculated via the Agrodrench
280 method, in which 5 mL of 1:3 TRV1:TRV2 suspensions were aliquoted to root crowns (Ryu et al., 2004). At
281 22 dpi, this process was repeated for a second dose of GAME-VIGS Agrodrench. At 41 days following the
282 initial dose, mature red tomatoes were harvested for *Colletotrichum* C9 inoculation and lesion size
283 measurement as previously described.
284
285 **Data analysis and software**
286
287 Statistical significance was determined via one-way Student's T-test or ANOVA with post-hoc
288 Tukey testing, as noted, using Microsoft Excel or R Studio version 2021.09.0 with R version 4.1.1, with the
289 "multcompView" package, as appropriate. Bar plots were generated in R with the "ggpubr" package.
290 Correlograms were generated, including determination of Pearson correlation coefficients, in R via the
291 "ggally" package. Scatterplots were generated in R via the "ggplot2" package. Heatmaps were generated
292 in R via the "pheatmap" package. Plasmid maps were generated in Geneious Prime version 2022.0.1.
293
294 **Results**
295
296 **Tissue extracts of an anthracnose-resistant, wild tomato accession convey fungicidal activity**
297 **against *Colletotrichum***
298
299 To evaluate the respective levels of anthracnose resistance in US28 and 95L368, ripe fruit were
300 harvested, inoculated with *Colletotrichum*, and scored for lesion diameter at 9 dpi (Fig. S2a). We observed
301 characteristic anthracnose lesions in US28 fruit, however 95L368 fruit were minimally symptomatic and in
302 total, displayed a 33-fold decrease in lesion size when compared to US28 (Fig. 1a-b). We sought to
303 determine whether the enhanced anthracnose resistance exhibited by ripe 95L368 fruit was the result of
304 antifungal, biochemical activity in fruit tissue. Utilizing whole supernatant, water-extracted, and methanol-
305 extracted fruit tissue samples supplemented onto PDA plates, we observed a restriction of mycelial growth
306 on plates supplemented with whole supernatant or methanol-extracted fraction, indicative of fungistatic
307 activity of polar compounds that accumulate in 95L368 fruit (Fig. 1c). To further validate these findings,
308 methanol extractions of US28 and 95L368 ripe fruit were separated via TLC, after which a suspension of

309 *Colletotrichum* C9 (3 x $10^6$ spores $mL^{-1}$) was sprayed onto the TLC plate. At 3-4 dpi, a zone of inhibition
310 around the polar phase of 95L368 fruit tissue extract was observed (Fig. S2b). Together, these findings
311 suggested that one or more polar compounds that accumulate in 95L368 red fruit possess fungistatic
312 activity against *Colletotrichum*, thereby contributing to anthracnose resistance.
313
314 **Ripe fruit tissue of the anthracnose-resistant accession 95L368 is enriched in the production of α-**
315 **tomatine and several α-tomatine derivatives**
316
317 To identify compounds that contribute to enhanced anthracnose resistance in 95L368, we
318 conducted a pilot study of phase-separated polar extracts of ripe US28 and 95L368 red fruit tissue. Tissue
319 extracts were separated via TLC as described, and the regions of the TLC plate corresponding to the zone
320 of inhibition of *Colletotrichum* growth (Fig. S2b) were analyzed via HPLC-MS. In a comparison of the total
321 ion chromatograms (TICs) generated for 95L368 and US28 samples we identified elution of several
322 steroidal glycoalkaloids (SGAs) in the retention time range of 20-25 minutes (Fig. 2a; Fig. S3). Additionally,
323 TICs of US28 red fruit displayed a peak at 19.9 minutes that was identified as esculeoside A (*m*/*z* =
324 1,270.61). Notably, this peak was absent in TICs of 95L368, which instead displayed strong peaks at 23.1
325 and 22.7 minutes, indicative of an enrichment of α-tomatine (*m*/*z* = 1,034.55) and the derivative
326 hydroxytomatine (*m*/*z* = 1,092.56), respectively.
327 We next sought to evaluate the distribution of several key SGAs in green, breaker (intermediate
328 ripening), and ripe red fruit. Methanol extractions of tissue samples from US28 and 95L368 fruit were
329 analyzed via matrix-assisted desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. In the
330 resulting spectrogram for US28, we observed signals (*m*/*z* ≈ 1,034 and 1,056), indicative of an accumulation
331 of α-tomatine ($C_{50}H_{83}NO_{21}$) and its sodium adduct ion in both green and breaker fruit (Fig. 2b). As expected,
332 these α-tomatine signals were also detected in breaker fruit, but not in red ripe fruit, while both breaker and
333 red fruit yielded signals (m/z ≈ 1,270 and 1,292) corresponding to the major and sodium adduct ions of
334 esculeoside A, consistent with the working model for SGA biochemistry in ripening tomato fruit.
335 Interestingly, an unidentified compound yielded suspected major (m/z ≈ 1,213) and sodium adduct (m/z ≈
336 1,236) ions, perhaps indicative of deacetoxylated esculeoside A or dehydroxylated esculeoside B
337 ($C_{56}H_{92}NO_{27}$; MW = 1,211.32), in both breaker and red fruit samples from US28. Analysis of the spectra for
338 green and breaker fruit tissue of 95L368 identified an accumulation of α-tomatine, however, in contrast to
339 US28, α-tomatine was also detected in ripe fruit tissue (Fig. 2c). Additionally, esculeosides were not
340 observed in the spectra for breaker and red fruit samples of 95L368; instead, these samples yielded strong
341 signals (m/z ≈ 1,093 and 1,115) identified as the major and sodium adduct ions for acetoxytomatine, the
342 most proximal derivative of α-tomatine in the SGA metabolic pathway.
343
344 **GAME genes are variably expressed in anthracnose-susceptible and -resistant cultivars**
345
346 Because we observed that fruit of the anthracnose-resistant 95L368 accumulated α-tomatine and
347 hydroxytomatine in both green and red fruit, whereas these compounds decline in ripe fruit of anthracnose-
348 sensitive US28, giving way to the derivative esculeosides, we surveyed the expression of GAME genes,
349 which encode enzymes in the SGA biosynthetic pathway, in both green and red fruit of US28 and 95L368.
350 Overall expression of *GAME4* and *GAME2* declined in the transition from green to red fruit, and in a
351 comparison to US28 green fruit, we observed 95L368 green fruit to exhibit 37.4- and 17.7-fold higher
352 expression of *GAME4* and *GAME2,* respectively (Fig. 2d). Interestingly, red 95L368 fruit were characterized
353 by 2.7-fold higher expression of *GAME31*, as well as a 4.6-fold depletion of *GAME5* transcripts, when
354 compared to red US28 fruit. This expression profile of GAME genes in anthracnose-resistant 95L368
355 supports our observation of enhanced accumulation of α-tomatine and acetoxytomatine in both green and
356 red fruit, as well as the depletion of esculeosides in red ripe fruit.
357

358 **SGA metabolic profiles correlate with anthracnose resistance in RILs developed from a cross**
359 **between US28 and 95L368**
360
361 Previous work in our laboratory utilized a tomato population developed from crosses between US28
362 and multiple different anthracnose-resistant breeding lines, including 95L368, to characterize the heritability
363 of anthracnose resistance (Stommel, 2001; Stommel and Haynes, 1998). Because anthracnose resistance
364 was determined to be a polygenic trait, we evaluated a population of twenty-eight recombinant inbred lines
365 (RILs), developed from a cross between US28 and 95L368, that exhibit variety in anthracnose susceptibility.
366 This population, as well as the parent lines US28 and 95L368, were surveyed for *Colletotrichum* resistance,
367 yielding a distribution of mean lesion diameters ranging from 0.39 to 11.33 mm (Fig. 3; Table S1). Next, we
368 profiled the SGA metabolomes of this population. Methanol extractions separated and analyzed via
369 UHPLC-HRMS yielded mean total SGA levels between 137.21 and 58,929.66 µg g dry weight$^{-1}$, with peaks
370 corresponding to α-tomatine, as well as peaks putatively identified as five SGA derivatives of α-tomatine:
371 multiple isomers of hydroxytomatine (*m*/*z* = 1,050.55); acetoxytomatine (*m*/*z* = 1,092.56); acetoxy-
372 dehydrotomatine (*m*/*z* = 1,090.54); esculeoside A (*m*/*z* = 1,270.61); and esculeoside B (*m*/*z* = 1,228.60)
373 (Table S2; Fig. S4). To evaluate the potential contributions of individual SGAs to anthracnose resistance,
374 we performed correlation analyses on anthracnose susceptibility and SGA levels. Due to variance in overall
375 anthracnose disease severity across planting blocks (Fig. S2; Table S1), lesion diameter values in each
376 planting block were transformed to "severity" values by normalizing to the maximum lesion size in that block.
377 Pearson correlation coefficients for severity and the $\log_{10}$-transformed SGA measurements conveyed
378 negative correlations ($-0.5 \leq R^2 \leq -0.68$) between disease severity and the SGAs α-tomatine,
379 hydroxytomatine, acetoxytomatine, and dehydro-acetoxytomatine, with hydroxytomatine levels yielding the
380 strongest correlation ($R^2 = -0.68$) (Fig. 4a-b; Fig. S5). Among the SGAs measured, hydroxytomatine,
381 acetoxytomatine, and dehydro-acetoxytomatine exhibited strong positive correlations ($0.73 \leq R^2 \leq 0.97$)
382 with each other, as did esculeoside A and esculeoside B ($R^2 = 0.69$).
383 To survey the distribution of anthracnose disease severity and accumulation of the individual SGAs
384 at the level of individual RILs and parent lines, we generated a heat map with RILs ordered by increasing
385 anthracnose severity. Interestingly, the six most anthracnose-resistant RILs (99L444, 99L391, 99L1-14,
386 99L394, 99L334, and 99L313) exhibited a pattern of relative enrichment in hydroxytomatine,
387 acetoxytomatine, and dehydro-acetoxytomatine. Conversely, nine most anthracnose-susceptible RILs
388 (99L375, 99L437, 99L453, 99L424, 99L314, 99L395, 99L418, 99L473, and 99L1-28) were relatively
389 depleted of these analytes and possessed relatively higher levels of the esculeosides (Fig. 4c). Of the SGAs
390 most positively correlated with anthracnose resistance, only hydroxytomatine has been characterized for
391 synthesis by a corresponding GAME gene (*GAME31*). To investigate whether modulated expression of
392 GAME genes is associated with enrichment or depletion of SGAs in RILs, we selected two highly
393 anthracnose-resistant (99L444 and 99L391) and -susceptible (99L453 and 99L375) RILs for GAME gene
394 expression analysis and observed variable enrichment of *GAME2* and *GAME31* transcripts in the selected
395 anthracnose-resistant RILs (Fig. S6a). Additionally, methanol extractions of US28, 95L368, and the four
396 selected RILs were tested in a plate growth inhibition assay using *Colletotrichum* strain C9, wherein
397 methanol extractions from 95L368 and the two anthracnose-resistant RILs displayed inhibitive activity,
398 restricting mycelial growth at the perimeter of the plate, in contrast to the extractions from anthracnose-
399 susceptible lines, which displayed no effect (Fig. S6b).
400
401 **Targeted silencing of *GAME31* or *GAME5* confers enhanced anthracnose defense**
402
403 Following our observations that tomato fruit with enhanced anthracnose resistance are enriched in
404 α-tomatine, hydroxytomatine, acetoxytomatine, and dehydro-acetoxytomatine, we theorized that targeted
405 gene silencing of select GAME genes would improve resistance to *Colletotrichum* in fruit of susceptible wild
406 type tomato. To that end, we employed a bipartite Virus-Induced Gene Silencing (VIGS) system.

407 Hypothesizing that silencing of *GAME31* would attenuate conversion of α-tomatine to hydroxytomatine,
408 thereby increasing α-tomatine levels, and that silencing of *GAME5* would reduce conversion of SGAs to the
409 esculeosides, we cloned a 406-bp region of *GAME31* coding sequence (CDS) and a 478-bp region of
410 *GAME5* CDS into the pYL156 TRV2 plasmid (Fig. S7). The resulting TRV1-TRV2 vector systems, in
411 addition to a TRV1-TRV2 empty vector system, were introduced to Micro Tom plants with developing fruit
412 via the Agrodrench method (Ryu et al., 2004). Ripe fruit were harvested for *Colletotrichum* infection, and at
413 7 dpi, we observed 4.3-fold and 3.4-fold reductions in mean lesion diameter in *GAME31*-VIGS and *GAME5*-
414 VIGS transformant fruit, respectively, accompanied by 3.6-fold and 2.4-fold reductions in target gene
415 expression (Figure 5).
416
417 **Discussion**
418
419 Anthracnose, caused by the hemibiotrophic fungal pathogen *Colletotrichum spp*., affects a wide
420 array of crop plants in the field and post-harvest, negatively impacting yield and threatening food security
421 in many growing regions across the globe. To date, there have been limited studies providing insights into
422 mechanisms of anthracnose resistance. Here, our work incorporating an anthracnose-resistant tomato
423 breeding line, as well as a population of 28 RILs derived from a cross between anthracnose-resistant and
424 -susceptible parentage, identifies a role for the secondary metabolites known as steroidal glycoalkaloids
425 (SGAs) in anthracnose defense in tomato. Our data illustrates a fungistatic activity against *Colletotrichum*
426 in red ripe fruit tissue extracts of the anthracnose-resistant breeding line 95L368, and through comparative
427 biochemical analysis of 95L368 and the anthracnose-susceptible cultivar US28, an enrichment in ripe
428 95L368 fruit of the SGAs α-tomatine and acetoxytomatine accompanying an absence of the end-product
429 esculeosides. Incorporating the population of RILs in a broader analysis of anthracnose resistance and
430 SGA metabolomic profiles, we identified positive correlations between anthracnose resistance and the α-
431 tomatine derivatives hydroxytomatine and acetoxytomatine, as well as dehydro-acetoxytomatine, a
432 dehydrotomatine derivative, respectively. Enhanced expression of GAME genes, which encode enzymes
433 in SGA biosynthesis, was observed in 95L368 and select anthracnose-resistant RILs, and targeted
434 silencing of *GAME31* and *GAME5* conferred enhanced anthracnose resistance in fruit of susceptible wild
435 type plants. Together, our findings introduce a role for several SGAs, previously shown to accumulate under
436 developmental control during the ripening process in tomato, in defense against anthracnose. Our data
437 further suggest that the transition of SGA profiles underpins the transition of *Colletotrichum* infections from
438 a quiescent state to necrotrophy during tomato fruit ripening.
439 Across our population of anthracnose-resistant and -susceptible parent and RIL lines we observed
440 a distribution of SGA accumulation (Fig. 4; Fig. S5; Table S2), as well as modulation of GAME gene
441 expression, namely enhanced expression of *GAME4*, *GAME2*, and *GAME31*, coincident with enhanced
442 anthracnose resistance (Fig. 3; Fig. S6). Additionally, targeted silencing of *GAME31* and *GAME5* conferred
443 enhanced levels of anthracnose resistance in wild type fruit (Fig. 5; Fig. S7). In tomato fruit, the transition
444 of *Colletotrichum* infections from a quiescent state to necrotrophy coincides with ripening, and our data
445 suggest that favorable variations in GAME gene expression elicit SGA metabolic profiles that enhance
446 anthracnose defense. The nature of the underlying regulation of GAME gene expression remains to be
447 elucidated. However, the ripening process in tomato fruit is characterized by a variety of changes within the
448 host tissue, such as the activation of production and signaling of the phytohormones abscisic acid, ethylene,
449 and jasmonic acid, and accordingly, phytohormones have been characterized for roles in the regulation of
450 SGA biosynthesis in tomato (Alkan et al., 2015; Cárdenas et al., 2015; Pesaresi et al., 2014; Quinet et al.,
451 2019). Research incorporating tomato mutant lines with constitutive production of jasmonic acid, as well as
452 exogenous treatment with methyl jasmonate (MeJA), have yielded enhanced levels of α-tomatine and
453 expression of GAME genes in root and leaf tissue; conversely, leaf tissue from mutant lines with attenuated
454 jasmonic acid production displayed lower levels of α-tomatine and several derivatives, as well as impaired
455 defense against the fungal pathogen *Fusarium oxysporum* (Montero-Vargas et al., 2018; Thagun et al.,

2016). Transcription factors known as jasmonate-responsive transcription factors of the ETHYLENE RESPONSE FACTOR (ERF) family (JREs) interact with JRE-binding elements in gene promoters and have been investigated for their role in the regulation of GAME gene expression. *GAME9*, also known as JRE4, is jasmonic acid-induced and expressed at high levels in various plant tissues, and interacts with the JRE-binding element in the 5' flanking regions of *GAME4* (Thagun et al., 2016; Yu et al., 2020). *JRE4* has also been shown to positively regulate the expression of multiple different GAME genes, and transgenic suppression of *JRE4* in tomato fruit conferred reductions in α-tomatine content in green fruit (Nakayasu et al., 2018; Thagun et al., 2016). Modulation of GAME gene expression, which impacts SGA production and anthracnose defense, may result from variable levels of jasmonic acid signaling and JRE transcription factor activity at key GAME genes. Therefore, to better understand the genetic mechanisms underlying anthracnose defense, future work should seek to assess endogenous jasmonic acid levels, jasmonic acid signaling activity, and GAME gene promoter identity and activity in anthracnose-resistant and -susceptible lines.

Multiple activities explaining the fungistatic activity of SGAs against pathogens have been proposed. Fungistatic activity of tomatidine against the human fungal pathogen *Candida albicans* was shown to occur through the ergosterol pathway, supporting a role for SGAs in membrane disruption of fungal pathogens (Dorsaz et al., 2017). *C. albicans* treated with tomatidine exhibited dose-dependent reductions of ergosterol, as well as inhibition of the sterol methyltransferase Erg6, and mutations in Erg6 were identified from a forward genetics approach utilizing tomatidine-resistant strains. Multiple studies have demonstrated the efficacy of α-tomatine in membrane disruption using liposomes, with reports of specificity of α-tomatine towards 3β-hydroxyl sterols such as ergosterol which, in free form, are present in lower amounts in tomato (Roddick and Drysdale, 1984; Steel and Drysdale, 1988; You and Kan, 2021). Alternatively, the induction of cell death in *Fusarium oxysporum* by α-tomatine was shown to occur via the generation of intracellular reactive oxygen species (ROS), likely through modulation of tyrosine kinase, G protein, and ATP synthase activities (Ito et al., 2007). The tetraose moiety of α-tomatine is important to its biological activity: Tomatinases, produced by various tomato pathogens, are capable of hydrolyzing one or more carbohydrate residues from this moiety, rendering α-tomatine less toxic and thereby contributing to virulence (Ökmen et al., 2013; Pareja-Jaime et al., 2008; Sandrock and VanEtten, 1998). Targeted manipulation of the SGA metabolic pathway and its metabolites may afford opportunities for durable host resistance against pathogen detoxification mechanisms.

We employed VIGS to silence expression of *GAME31* and *GAME5*, respectively targeted to reduce the conversion of α-tomatine to hydroxytomatine and the production of the esculeosides, with both strategies conferring enhanced anthracnose resistance (Fig. 5; Fig. S7). Our results demonstrated fungistatic activities of red tomato fruit tissue extracts possessing high levels of α-tomatine and the derivatives hydroxytomatine, acetoxytomatine, and dehydro-acetoxytomatine, and positive correlations between the accumulation of these compounds and anthracnose resistance in red fruit (Fig. 1-2; Fig. S2-S4; Fig. 4; Table S2). One or more of these derivatives may possess antifungal activity similar to that of α-tomatine, however, no studies have individually examined these α-tomatine derivatives for their antifungal activities. Therefore, future studies might seek to isolate these compounds for structural evaluation of their fungistatic activities against *Colletotrichum* and other fungal pathogens of tomato and other crop species. Furthermore, the targeted manipulation of GAME gene expression presented here can be expanded to develop tomato breeding lines with stable, targeted enhancement or suppression of GAME gene expression, with the purpose of generating high levels of SGAs conferring enhanced resistance to *Colletotrichum* and other tomato pathogens. Together, our data demonstrates a role for SGAs, including α-tomatine and certain of its derivatives, as well as GAME genes including *GAME31* and *GAME5*, in anthracnose defense in tomato fruit, findings that can be utilized to further optimize management of tomato pathogens that impact yield.

**Supplementary Data**

505
506 Fig. S1. Simplified schematic of the steroidal glycoalkaloid (SGA) metabolic pathway
507 during tomato fruit ripening.
508 Fig. S2. *Colleotrichum* resistance in the tomato accession 95L368.
509 Fig. S3. 95L368 accumulates enhanced levels of SGAs.
510 Fig. S4. Representative extracted ion chromatograms (EICs) for detected SGAs.
511 Fig. S5. Mean detected SGAs vs. mean anthracnose disease severity.
512 Fig. S6. Anthracnose-resistant and –susceptible RILs exhibit variable GAME gene
513 expression and *Colletotrichum* growth inhibition.
514 Fig. S7. Transient silencing of *GAME* genes via VIGS.
515 Table S1. Anthracnose resistance distribution among parent lines and RILs.
516 Table S2. SGA measurements (ug $gDW^{-1}$) among parent lines and RILs.
517 Table S3. PCR primers utilized in this study.
518

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520

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525

526 **Author Contributions**

527

528 Fabian, M.L. and Zhang, C. contributed equally to this study.

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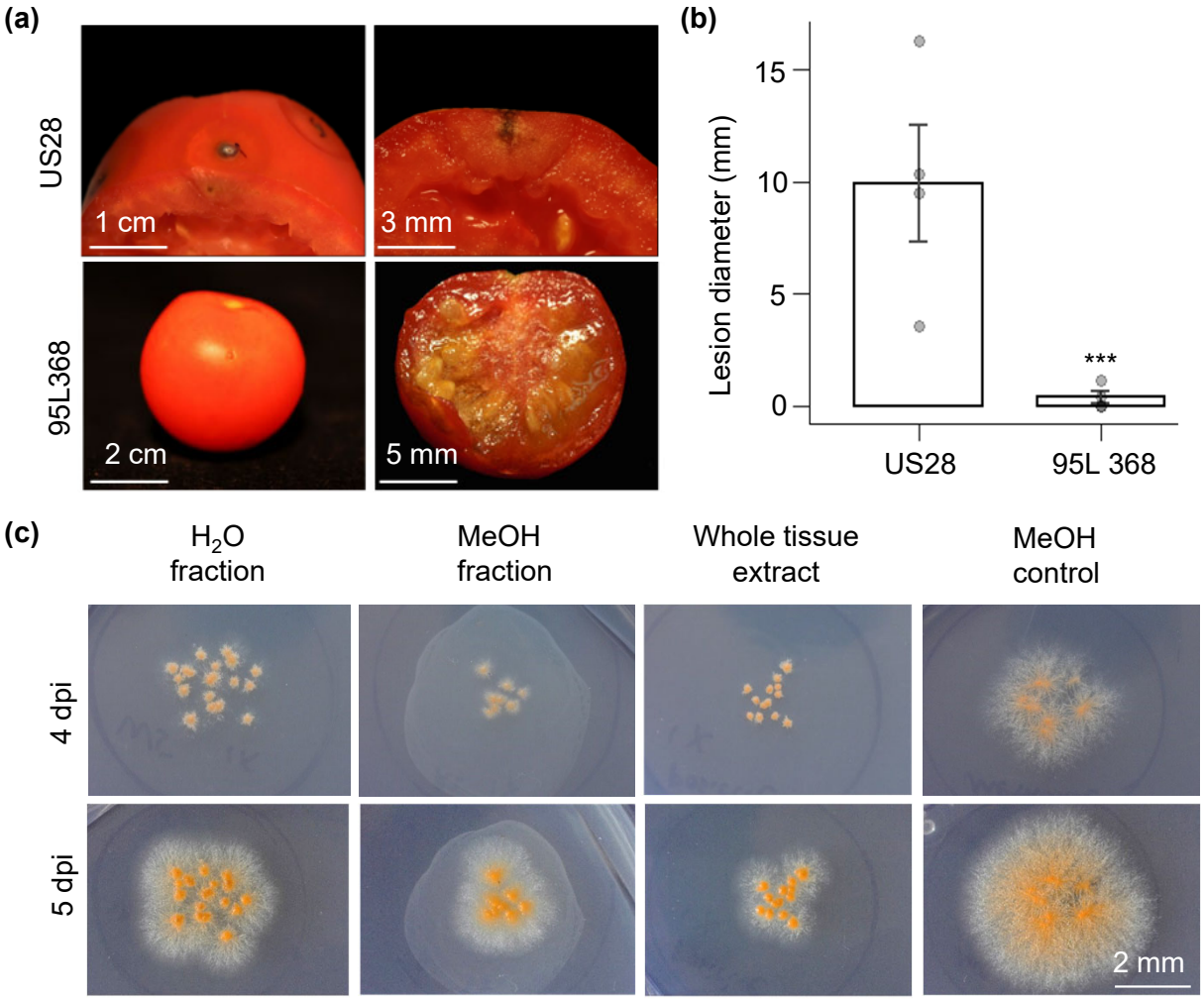


**Figure 1.** Fruit tissue extracts from an anthracnose-resistant tomato accession exhibit fungistatic activity against *Colleotrichum*. (a) Representative images (9 dpi) of anthracnose-susceptible (US28, top) and resistant (95L368, bottom) tomato fruit following inoculation with *Colletotrichum*. (b) Lesion diameter following *Colletotrichum* infection. Ripe fruit were harvested and inoculated with *Colletotrichum,* with lesion size measured at 9 dpi. Data points represent average lesion size from each of 4 planting blocks per line, with error bars representing SEM. Asterisks represent statistical significance (one-way Student's T-test). (c) *Colletotrichum* growth inhibition plate assay. $H_2O$- or MeOH-fractionated and whole extracts from 95L368 fruit tissue were spotted on PDA plates before inoculation with *Colletotrichum*.

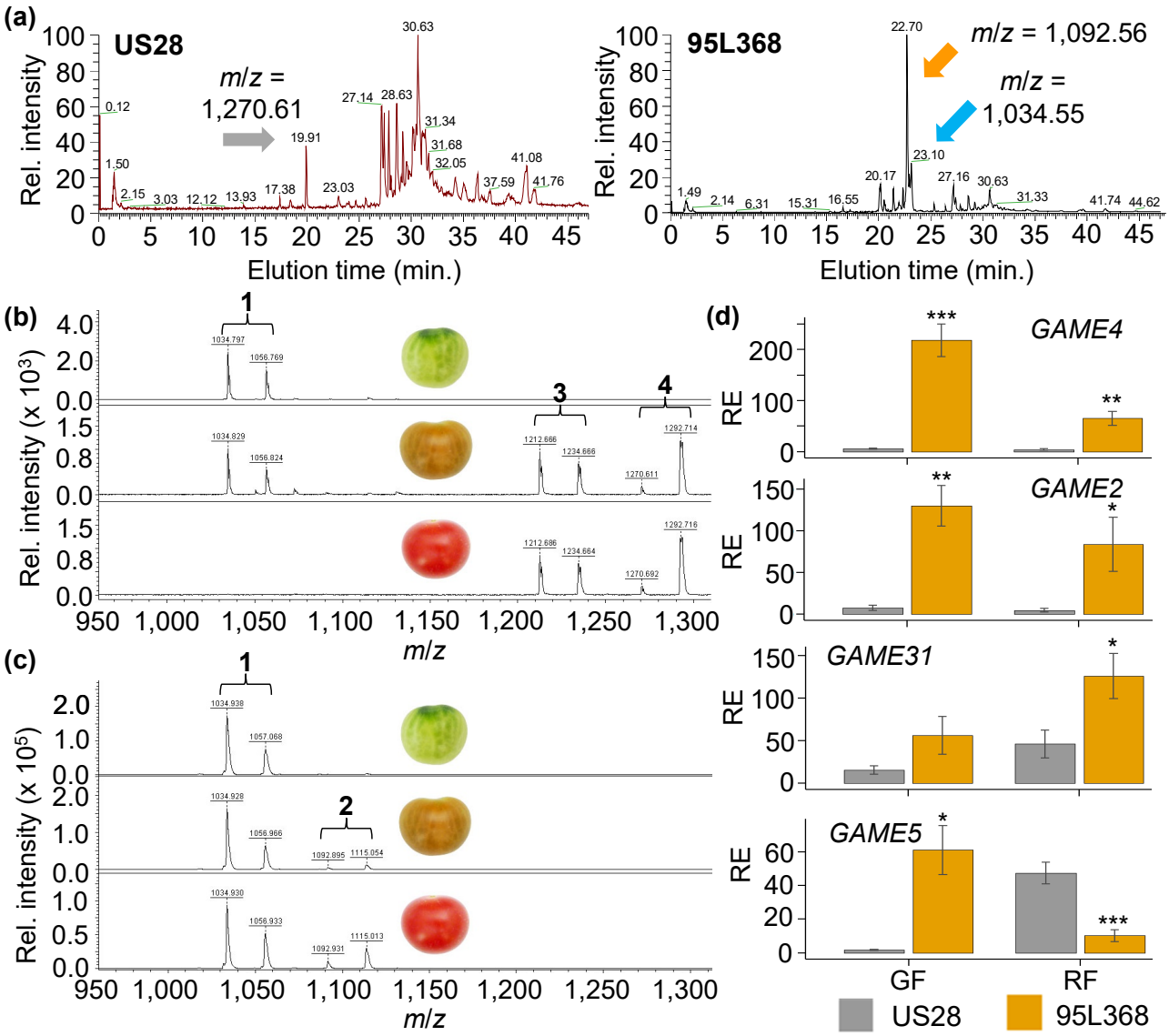


**Figure 2.** SGA metabolic and *GAME* transcript profiles in green and ripe red fruit of anthracnose-susceptible and –resistant tomato cultivars. (a) Representative TICs from electrospray negative ionization HPLC-MS of ripe red fruit tissue samples of US28 (left) and 95L368 (right). Arrows indicate peaks corresponding to esculeoside A (grey), α-tomatine (orange), and hydroxytomatine (blue). (b) and (c) Representative chromatograms from MALDI-TOF MS analysis of US28 (b) and 95L368 (c) fruit tissue by ripening stage. From top to bottom: green fruit, breaker fruit, and red fruit. From left to right: (1) α–tomatine; (2) acetoxytomatine; (3) unknown compound; and (4) esculeoside A. (d) Expression analysis of GAME gene expression in US28 and 95L368 in green (GF, left) and red (RF, right) fruit. qRT-PCR was conducted with 2 technical replicates of 3 biological replicates per sample, in 3 experiments. RE indicates relative expression, asterisks indicate statistical significance (1-way Student's T-test), and error bars represent SEM.

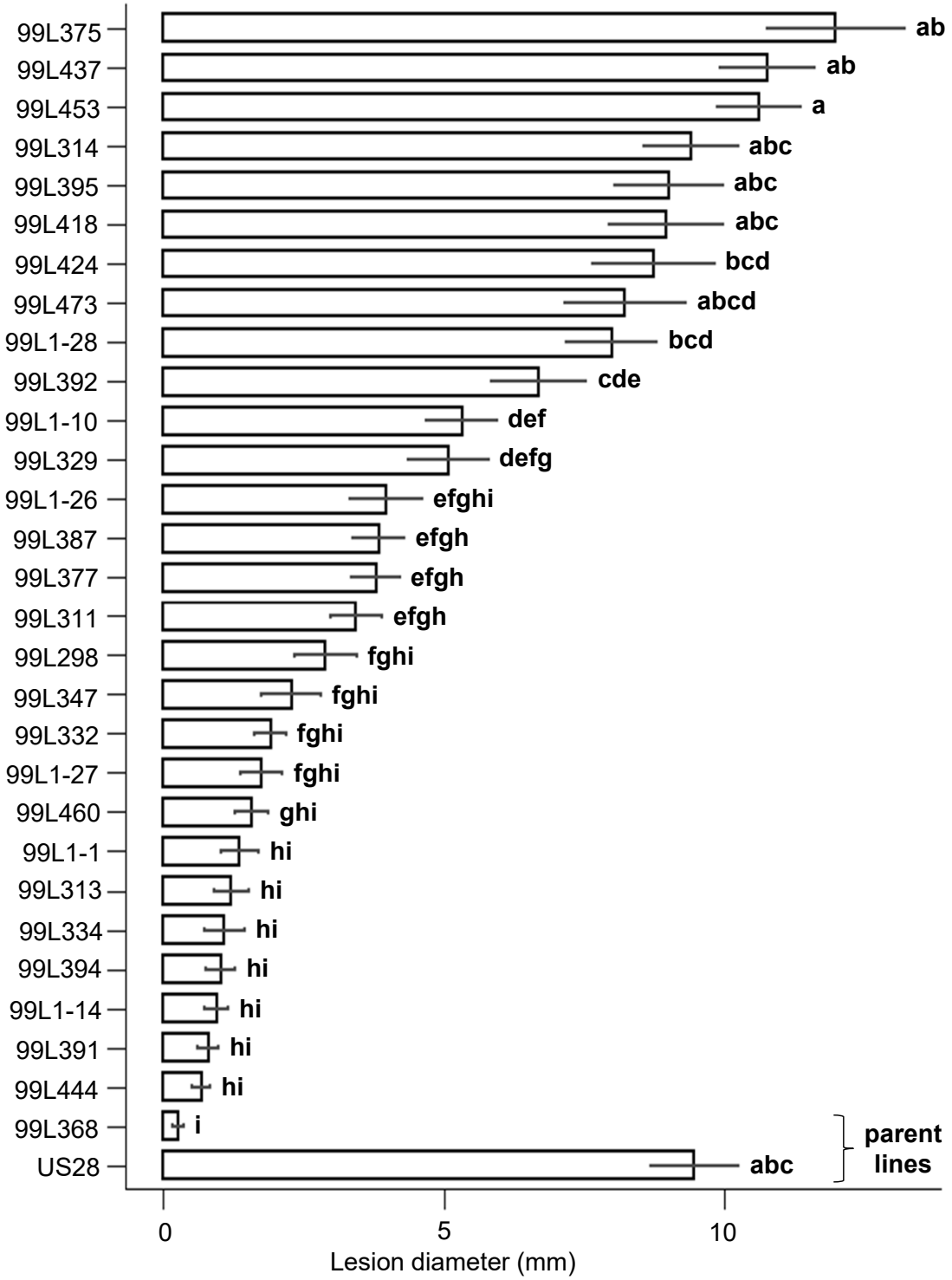


**Figure 3.** Anthracnose resistance distribution among parent lines and RILs. Ripe red fruit were harvested and inoculated with *Colletotrichum* and incubated in greenhouse conditions, with lesion diameter measured at 9 dpi. Data points represent mean lesion size from each of 4 planting blocks per line, with error bars representing SEM. Statistical significance was determined via ANOVA with post-hoc Tukey testing.

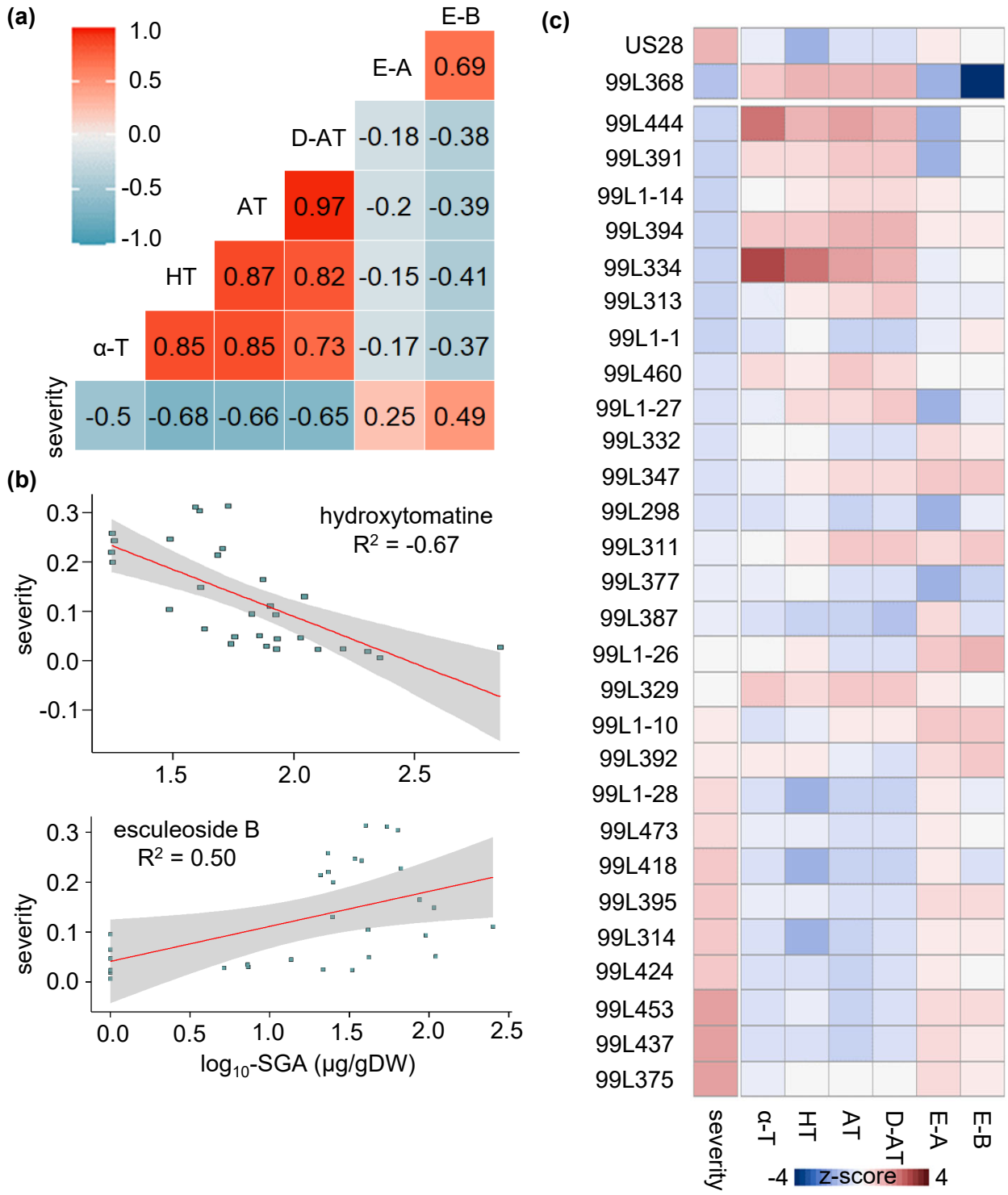


**Figure 4.** SGAs variably correlate to anthracnose resistance in the parent lines and RIL population. (a) Pearson correlogram of total anthracnose disease severity and SGA levels. (b) Scatterplot of anthracnose severity vs. hydroxytomatine (upper) and esculeoside A (lower) levels with general linear model (GLM) trendline and 95% C.I. Each point corresponds to an individual parent line or RIL. (c) Heat map of z-score-transformed anthracnose severity and SGA levels by parent line and RILs. SGA levels for (a) - (c) are $\log_{10}$-transformed. α–T, α -tomatine. HT, hydroxytomatine. AT, acetoxytomatine. D-AT, dehydro-acetoxytomatine. E-A, esculeoside A. E-B, esculeoside B.

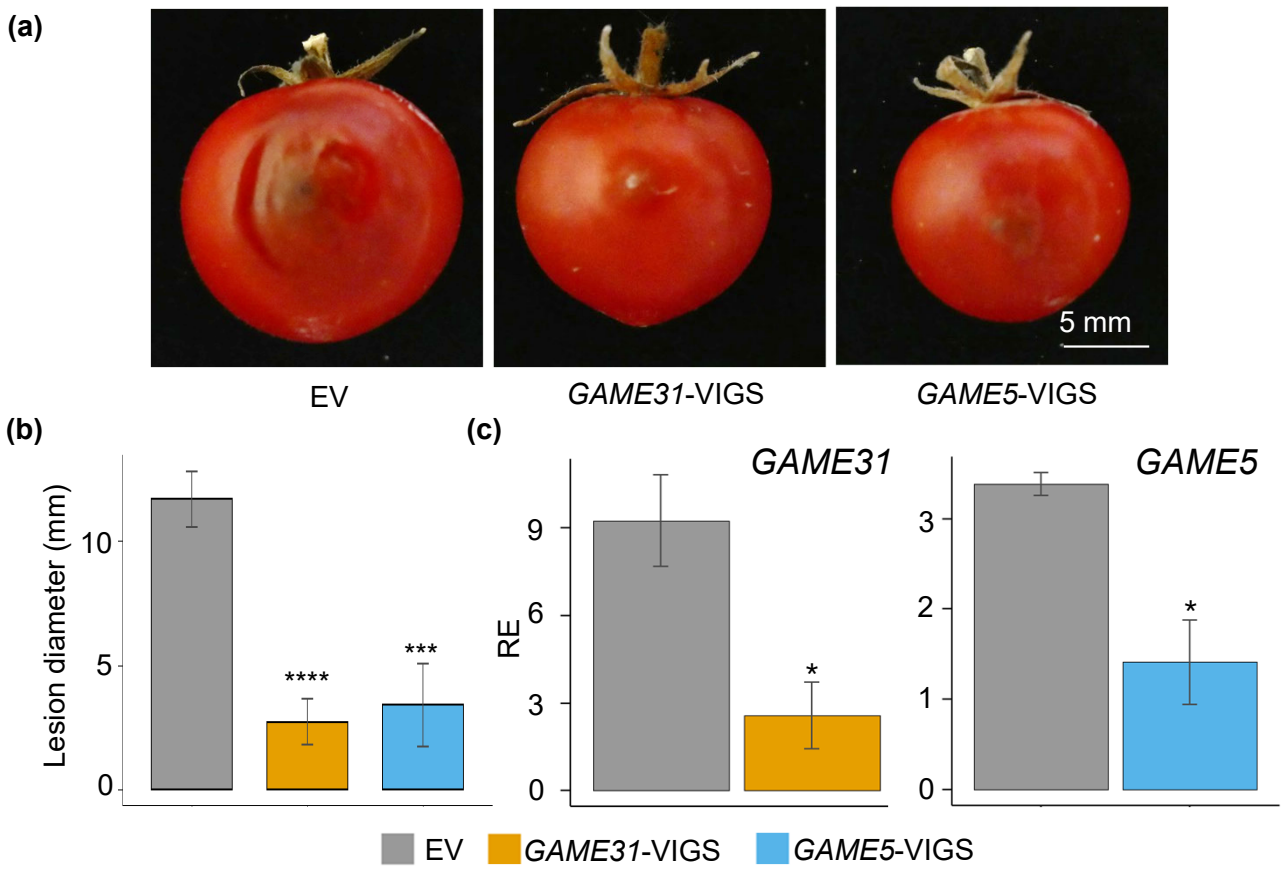


**Figure 5.** Transient, targeted silencing of *GAME31* and *GAME5* confer enhanced anthracnose resistance in wild type fruit. Bipartite VIGS vectors targeting silencing of *GAME31* or *GAME5* were introduced to Micro Tom plants (n = 2 per treatment) presenting immature green fruit via the Agrodrench method, and mature red fruit were harvested for *Colletotrichum* infection and gene expression via qRT-PCR. (a) Representative images of infected fruit. (b) Lesion diameter of infected fruit ($n \geq 9$). (c) Expression of *GAME31* (left) and *GAME5* (right). Samples from infected fruit were collected at 7 dpi and processed for RNA extraction. For each target, qRT-PCR was conducted with 2 technical replicates of 3 biological replicates per treatment. EV, empty vector control. RE indicates relative expression, asterisks indicate statistical significance (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, 1-way Student's t-test) and error bars indicate SEM.