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4 **Selinene volatiles are essential precursors for maize defense promoting**

5 **fungal pathogen resistance**

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16 **Running title:** Selinene volatiles underlay antifungal defenses

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18 **One Sentence Summary:** Maize terpene synthase 21 encodes a β-selinene

19 synthase enabling the production of antifungal defenses.

20 **List of Author contributions:**

21 E.A.S., A.H. and Y.D. conceived the original screening and research plans; Y.D.

22 performed most of the experiments; P.W. provided technical assistance to Y.D.;

23 Y.D., T.G.K., C.A.M.R., J.L.S., A.E.L. designed the experiments and analyzed

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46   **Abstract**

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To ensure food security, maize (*Zea mays*) is a model crop for understanding useful traits underlying stress resistance. In contrast to foliar biochemicals, root defenses limiting the spread of disease remain poorly described. To better understand below-ground defenses in the field, we performed root metabolomic profiling and uncovered unexpectedly high levels of the sesquiterpene volatile β-selinene and the corresponding non-volatile antibiotic derivative, β-costic acid. The application of metabolite-based Quantitative Trait Loci (mQTL) mapping using bi-parental populations, genome wide association studies, and near-isogenic lines (NILs) enabled the identification of terpene synthase 21 (*ZmTps21*) on chromosome 9 as a β-costic acid pathway candidate gene. Numerous closely examined β-costic acid deficient inbred lines were found to harbor *Zmtps21* pseudo genes lacking conserved motifs required for farnesyl diphosphate (FPP) cyclase activity. For biochemical validation, a full length *ZmTps21* was cloned, heterologously expressed in *E. coli* and demonstrated to cyclize FPP yielding β-selinene as the dominant product. Consistent with microbial defense pathways, *ZmTps21* transcripts strongly accumulate following fungal elicitation. Challenged field roots containing functional *ZmTps21* alleles displayed β-costic acid levels over 100 μg g^{-1} FW, greatly exceeding *in vitro* concentrations required to inhibit the growth of five different fungal pathogens and rootworm larvae (*Diabrotica balteata*). *In vivo* disease resistance assays, using *ZmTps21* and *Zmtps21* NILs, further support the endogenous antifungal role of selinene-derived metabolites. Involved in the biosynthesis of non-volatile antibiotics, *ZmTps21* exists as a useful gene for germplasm improvement programs targeting optimized biotic stress resistance.

Introduction

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75 Plants are protected from a broad range of harmful biotic agents by initial

76 perception events, signal transduction cascades and the elicitation of defense

77 metabolism (Vanetten et al., 1994; Harborne, 1999; Dangl et al., 2013; Huffaker

78 et al., 2013). In maize (*Zea mays*), seedlings are largely protected from attack by

79 a complex suite of hydroxamic acid-based defenses, termed benzoxazinoids

80 (BX), responsible for resistance to diverse threats spanning fungal pathogens

81 and herbivores including Northern corn leaf blight (*Setosphaeria turtica*) and the

82 European corn borer (ECB; *Ostrinia nubilalis*) (Beck et al., 1957; Couture et al.,

83 1971; McMullen et al., 2009). Sixty years of research has resulted in a nearly

84 complete metabolic and genetic BX pathway in maize involving over a dozen

85 individual enzymes and metabolites (Frey et al., 2009; Meihls et al., 2013;

86 Handrick et al., 2016). Additionally, diverse terpenoids and underlying terpene

87 synthases (Tps) have also been demonstrated to play important protective roles

88 (Degenhardt, 2009; Schmelz et al., 2014). As indirect defenses, herbivore-

89 elicited terpene volatiles can function as diffusible signals to attract natural

90 enemies, such as parasitoids and entomopathogenic nematodes, to above- and

91 below-ground insect pests, respectively (Rasmann et al., 2005; Schnee et al.,

92 2006).

93   Of the many biosynthetic classes of natural products, terpenoids are the

94 most structurally diverse with well over 25,000 established compounds. In

95 addition to roles as phytohormone signals, specialized terpenoids mediate inter-

96 organism interactions and serve as chemical barriers (Gershenzon and

97 Dudareva, 2007). In maize, terpene olefins are nearly ubiquitous components of

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98   induced above and below-ground volatile emissions acting as indirect plant

99   defenses following biotic stress (Turlings et al., 1990; Degenhardt, 2009;

100   Degenhardt et al., 2009; Kollner et al., 2013). Maize terpene olefins can also

101   serve as precursors for localized production of non-volatile antibiotic terpenoid

102   defenses (Schmelz et al., 2014). While often undetectable at the level of volatile

103   pathway intermediates, the inducible accumulation of non-volatile terpenoid end-

104   products can limit the damage caused by fungi, herbivores and oxidative

105   stresses (Harborne, 1999; Ahuja et al., 2012). Despite significant advances,

106   continuing discoveries in maize reveals that our collective knowledge of

107   biochemical defenses and pathway genes responsible for mitigating crop stress

108   remains incomplete.

109   Decades of intensive research in related Poaceous crops, such as rice

110   (*Oryza sativa*), has revealed multiple pathways of inducible labdane-related

111   diterpenoids including momilactones, oryzalexins, and phytocassanes that

112   underlay protective responses to biotic and abiotic stress (Schmelz et al., 2014).

113   More recently, complex arrays of acidic terpenoids have been detected in maize

114   and include sesquiterpenoids derived from β-macrocarpene and diterpenoids

115   derived from *ent*-kauranes, termed zealexins and kauralexins, respectively

116   (Huffaker et al., 2011; Schmelz et al., 2014). From a biosynthetic and pathway

117   perspective, maize genes underlying the production of antifungal agents remain

118   largely unknown. In the case of maize diterpenoid defenses, a specific *ent*-

119   copalyl diphosphate synthase (Anther ear 2; ZmAn2), is the only enzyme

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120   demonstrated *in planta* essential for kauralexin biosynthesis (Vaughan et al.,

121   2015).

122   To uncover further defense pathways, we employed targeted metabolomic

123   profiling on field grown maize roots naturally exposed to combinations of

124   herbivores and pathogens (Baldwin, 2012). Curiously, high levels of rarely

125   encountered eudesmane sesquiterpenoids including β-selinene and β-costic acid

126   dominated the chemical profiles of many samples. While not previously

127   associated with maize, β-costic acid is known from the Asteraceae family,

128   including false yellowhead (*Dittrichia viscosa*) and costus (*Saussurea costus*),

129   and has been utilized in extracts for potent antibiotic activities against diverse

130   organisms (Rao and Alvarez, 1981; Wu et al., 2006; Katerinopoulos et al., 2011).

131   Despite the diverse phylogenetic occurrence in nature, a specific pathway

132   predominantly leading to β-costic acid has not been described in plants. To

133   explore the maize β-costic acid pathway, combined genetic mapping approaches

134   with the intermated B73 x Mo17 (IBM) population of recombinant inbred lines

135   (RILs) (Lee et al., 2002), the Goodman diversity panel (Flint-Garcia et al., 2005),

136   and IBM near-isogenic lines (NILs) (Eichten et al., 2011) were used for

137   metabolite-based Quantitative Trait Loci (mQTL) mapping. Biochemical

138   characterization of the mQTL-identified Tps candidate utilized heterologous

139   expression in *E. coli* to confirm identification of a comparatively product-specific

140   β-selinene synthase. Transcript expression and metabolite analyses following

141   elicitation with multiple pathogens and western corn rootworm (WCR, *Diabrotica*

142   *virgifera virgifera*) larvae (Gray et al., 2009; Meinke et al., 2009; Miller et al.,

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143   2009; Spencer et al., 2009; Tinsley et al., 2013) were used to assess pathway

144   activation. Concentrations of β-costic acid below those detected in field tissues

145   were then used to examine *in vitro* antibiotic activity against 5 fungal species.

146   Similarly, NILs were used to investigate *in vivo* root resistance following

147   challenge with *Fusarium verticillioides* and *Fusarium graminearum*. Collectively

148   our results support the existence of a previously unrecognized β-costic acid

149   pathway in maize that contributes to fungal pathogen resistance.

150

151   **RESULTS**

152   **Identification of α and β-selinene derived products as inducible maize**

153   **sesquiterpenoids that can influence generalist root herbivores.** Our previous

154   investigation of maize responses following stem herbivory and fungal elicitation

155   enabled the discovery of two distinct biosynthetic classes of inducible acidic

156   terpenoids (Huffaker et al., 2011; Schmelz et al., 2011). Similarly, experiments

157   examining maize root defenses elicited by banded cucumber beetle (*Diabrotica*

158   *balteata*) larvae and *F. verticillioides* infection confirmed shared responses in

159   diverse tissue types (Vaughan et al., 2015). Given that predominant defenses

160   change over ontogeny and that controlled lab experiments do not capture the full

161   suite of biotic stresses in nature (Kollner et al., 2004; Baldwin, 2012), we sought

162   to expand our targeted metabolomic analyses to roots in the context of natural

163   biotic challenge (Schmelz et al., 2004). As expected, mature visibly-necrotic roots

164   of field-challenged maize lines including hybrid sweet corn (var. Golden Queen)

165   and the inbred Mo17 contained zealexins (Fig. 1A); however, chemically

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166 extracted samples unexpectedly also contained α-selinene, β-selinene, β-costol,

167 α-costic acid and β-costic acid (Fig. 1; Supplemental Fig. S1). In volatile

168 collections of live Mo17 root emissions, α-selinene, β-selinene (Fig. 2) and the

169 aldehyde β-costal (Supplemental Fig. S1) were likewise detectable. As the major

170 analyte, live field-collected Mo17 roots displaying visible necrosis emit

171 predominantly β-selinene (Fig. 2). In contrast, β-selinene emission is absent in

172 B73 roots; however, production reappears in select B73 x Mo17 RILs, for

173 example IBM0287 (Fig. 2). Similar volatile emission results are observed in live

174 Mo17 stems following inoculation with the necrotrophic fungal pathogen

175 (*Cochliobolus heterostrophus*), commonly known as southern leaf blight (SLB)

176 (Fig. 2). Consistent with root metabolite patterns, the reference genome inbred

177 B73 (Schnable, 2009) remains void of α and β-selinene stem volatiles under

178 identical conditions (Fig. 2). Qualitative metabolite differences between B73,

179 Mo17 and select RILs provides empirical evidence for genetic variation in

180 selinene biosynthesis and encourages the use of genetic mapping resources

181 (Lee et al., 2002).

182   Our quantification of unexpectedly high levels of β-selinene and β-costic

183 acid in field collected maize roots was paired with casual field observations of

184 adult *D. balteata* beetles on leaves. Given the broad host range of *D. balteata*

185 larvae (Saba, 1970) and pest pressures exerted by western corn rootworm

186 (WCR: *Diabrotica virgifera virgifera*) larvae including the promotion of secondary

187 disease (Flint-Garcia et al., 2009; Gray et al., 2009), we conducted controlled

188 *Diabrotica-maize* interaction experiments. In growth chamber assays, tissue

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189 extracts of roots revealed both β-selinene and β-costic acid following damage by

190 WCR larvae (Fig. 2). Given the high levels of selinene-derived metabolites

191 observed in field-collected roots, additional assessments of WCR and *D. balteata*

192 preference and performance were conducted on larvae. For both *Diabrotica*

193 species, we observed no influence of exogenously applied β-costic acid on root

194 preference but found a significant inhibitory role of β-costic acid on *D. balteata*

195 performance (Fig. 2)*.*

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197 **Combined linkage and association mapping identifies maize terpene**

198 **synthase *ZmTps21* as a candidate biosynthetic gene.** β-selinene has been

199 previously detected in the volatile profiles of pathogen-challenged maize tissue;

200 however, the biosynthetic source and physiological function(s) have not been

201 elucidated (Becker et al., 2014). Given our observation that selinene-derived

202 pathway products can predominate in maize under specific conditions, we sought

203 to identify the gene(s) responsible. We first employed the IBM-RILs for mQTL

204 mapping. As a predictable non-volatile pathway end product, β-costic acid levels

205 were analyzed in naturally challenged roots of 216 IBM RILs (Supplemental

206 Table S1). Composite Interval Mapping (CIM) placed the locus in Bin 9.05 (Fig.

207 3) (Gardiner et al., 1993). For comparative purposes, the IBM-RIL data was also

208 explored using 173,984 SNPs and association mapping via a General Linear

209 Model (GLM) (Bradbury et al., 2007) and Unified Mixed Linear Model (MLM) (Yu

210 et al., 2006). All approaches supported a single statistically significant locus on

211 chromosome 9 (Fig. 3, Supplemental Fig. S2). Additionally we performed an

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212   elicited metabolite-based Genome-Wide Association Study (mGWAS) using β-

213   costic acid levels in greenhouse grown inbreds from the Goodman diversity panel

214   (Flint-Garcia et al., 2005). Similarly, we detected a single statistically significant

215   locus on chromosome 9 (Fig. 3). An independent mGWAS replication conducted

216   with field grown plants yielded an identical result (Supplemental Fig. S2).

217   Correspondence of physical QTL coordinates identified with IBM RILs and the

218   replicated GWAS results (Fig. 3, Supplemental Fig. S2) robustly supported a

219   single narrow locus controlling maize β-costic acid levels.

220   For additional confirmation, select B73×Mo17 near isogenic lines (NILs)

221   were analyzed following stem elicitation (Eichten et al., 2011). B73 chromosomal

222   segments introgressed into Mo17 dominating lines (specifically m012, m048,

223   m050 and m062) were each deficient in the production of β-costic acid (Fig. 3). In

224   contrast, β-costic acid production in NILs with introgressions of the Mo17 allele

225   into the B73 genetic background (lines b047, b055, b069, and b157) were similar

226   to Mo17 (Fig. 3, Supplemental Fig. S3), confirming existence of the β-costic acid

227   associated locus in Bin 9.05. Further analyses of additional NIL lines (b022,

228   b033, m002, m065, and m092) narrowed the locus to 13 predicted genes

229   isolated on bacterial artificial chromosome (BAC) clones, AC213878 and

230   AC204415 (Fig. 3). Of the remaining candidates, only a single uncharacterized

231   gene (GRMZM2G011151) displayed significant sequence homology with known

232   terpene synthases. Supportively, detailed examination of 3 independent

233   association mapping results likewise demonstrated the presence of highly

234   significant SNPs associated with the Tps candidate (GRMZM2G011151), termed

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235   *ZmTps21* (Supplemental Fig. S2). Genomic structure analysis of B73 *Zmtps21*

236   revealed three predicted exons encoding a 297 amino acid protein lacking the

237   conserved Tps catalytic domains, namely the DDXXD and RXR motifs (Fig. 3),

238   which are essential for function (Chen et al., 2011). Collectively, these findings

239   made B73 *Zmtps21* a parsimonious inactive β-selinene synthase pseudogene

240   candidate meriting further examination.

241   In an attempt to isolate the Mo17 *ZmTps21* cDNA sequence, early RT-

242   PCR trials with primers based upon B73 *Zmtps21* cDNA failed due to nucleotide

243   polymorphisms. Eventually a segment near the Mo17 *ZmTps21* 5'-end of

244   genomic DNA of was obtained by a PCR and sequenced. Alignments revealed

245   that the segment near the Mo17 *ZmTps21* 5'-end DNA fragment shared high

246   sequence similarity to that of B73 *Zmtps21*. Therefore, the 5'-end cDNA

247   sequence of Mo17 *ZmTps21* was obtained by RT-PCR and extended by PCR

248   with rapid amplification of cDNA ends (rPCR) using a cDNA library to obtain the

249   full-length Mo17 *ZmTps21* cDNA (Fig. 3; Supplemental Fig. S4). The deduced

250   amino acid sequence of the open reading frame contained the conserved

251   terpene synthase domains including the DDXXD (residues 325 to 329) and RXR

252   (residues 288 to 290) motifs (Supplemental Fig. S4). The amino acid sequence

253   of ZmTps21 resembles (<60% identity) those of other plant sesquiterpene

254   synthases and shares less than 40% sequence identity with previously

255   characterized maize sesquiterpene synthases, such as ZmTps6, ZmTps10,

256   ZmTps11, and ZmTps23 (Supplemental Fig. S4). With only 30% identity at the

257   amino acid level, Mo17 ZmTps21 is even more distantly related to the *Ocimum*

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258   *basilicum* sesquiterpene synthase (SES) which produces detectable levels of β-

259   selinene as part of a complex blend (Supplemental Fig. S4) (Iijima et al., 2004).

260   To understand the extent of genetic variation in *ZmTps21* alleles, we

261   examined 15 commonly investigated inbreds. *ZmTps21* genomic sequences

262   were isolated by PCR using primers based on the B73-*Zmtps21* and Mo17-

263   *ZmTps21* genome sequences, respectively (Supplemental Table S1). Sequence

264   analyses demonstrated that the *Zmtps21* alleles from B73-like lines (Ki3, M37W,

265   MS71, M162W, CML247, Ki11, Mo18W) share >98% DNA sequence identity and

266   basic genome structure, whereas Mo17-like *ZmTps21* alleles (Hp301, TX303,

267   Oh43, Oh7B, Ky21 and W22) contain six exons and share higher than 98%

268   sequence identity at the amino acid level (Supplemental Fig. S5 and S6). These

269   results support the hypothesis that B73-like inbred lines share a common

270   mutation ancestry.

271

272   ***In vitro* assays demonstrate that ZmTps21 is a largely product-specific β-**

273   **selinene synthase.** ZmTps21 lacks a predicted N-terminal transit peptide

274   suggesting that the enzyme is not targeted to plastids as is typical of

275   monoterpene and diterpene synthases, but instead remains cytosolic consistent

276   with predictions for a sesquiterpene synthase (Gershenzon, 1999). To obtain

277   additional support for the hypothesis that Mo17 ZmTps21 is a β-selinene

278   synthase, heterologous expression was performed in *E. coli* and the resulting

279   protein extract was incubated with the precursor substrate (*E,E*)-farnesyl

280   diphosphate (FPP). β-selinene is the dominant product observed by GC-MS

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281 along with several other minor sesquiterpene olefins, including α-selinene and β-

282 elemene (Fig. 4). Thus *ZmTps21* encodes a selinene synthase with predominant

283 β-selinene product specificity that includes α-selinene as a minor product

284 consistent with the olefin and oxygenated metabolite ratios observed *in planta*

285 (Fig. 1, Supplemental Fig. S1). Injection of the ZmTps21 reaction products on a

286 GC column at different temperatures revealed that the β-elemene present is due

287 to a Cope rearrangement of germacrene A (Supplemental Fig. S7 and S8) (de

288 Kraker et al., 2001). Germacrene A also is a neutral reaction intermediate of the

289 tobacco (*Nicotiana tabacum*) enzyme 5-*epi*-aristolochene synthase (TEAS)

290 responsible for the pathogen-elicited biosynthesis of capsidiol (Cane, 1990;

291 Starks et al., 1997). The enzymatic protonation of germacrene A leads to the

292 eudesmane carbocation further converted to 5-*epi*-aristolochene. Given that β-

293 selinene is simply formed by a deprotonation of a eudesmane carbocation, it

294 likely that the reaction catalyzed by ZmTps21 also includes the formation and

295 protonation of germacrene A. A sequence comparison of ZmTps21 with TEAS

296 and other Tps able to protonate neutral reaction intermediates demonstrate that

297 the amino acids of the catalytic triad involved in the protonation reaction are

298 conserved (Starks et al., 1997) (Supplemental Fig. S4). Curiously, two ZmTps21

299 mutants with altered C-termini obtained as cloning artifacts produced only

300 germacrene A (Supplemental Fig. S7 and S8), suggesting additional influence of

301 the C-terminus on the protonation reaction and specificity of the final product.

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303 ***ZmTps21* transcripts are pathogen inducible and correspond with β-costic**

304 **acid accumulation.** To examine endogenous patterns, we compared Mo17

305 *ZmTps21* expression with established *ZmTps6/11* expression associated with

306 zealexin biosynthesis (Kollner et al., 2008; Huffaker et al., 2011). Similar to

307 *ZmTps6/11*-zealexin relationships, *ZmTps21* transcripts and β-costic acid are

308 barely detectable in control tissues and not significantly elicited by mechanical

309 damage alone (Fig. 5). After elicitation with heat-killed *Fusarium* hyphae,

310 *ZmTps6/11* transcripts reached maximal levels at day 1 while *ZmTps21*

311 transcripts levels continued to accumulate for an additional day (Fig. 5). Zealexin

312 A1 was readily detectable at day 1 and continued to increase over the 4 days

313 while β-costic acid accumulation was first detected at day 2 reaching similar

314 levels at day 4 (Fig. 5). Thus, *ZmTps21* transcripts and product accumulation

315 display longer-term and temporally layered elicitation kinetics alongside the

316 zealexin biosynthetic pathway.

317   To examine whether *ZmTps21* transcripts and β-costic acid levels change

318 specifically in response to aggressive pathogens such as C. *heterostrophus* and

319 *F. verticillioides* or whether the response also follows opportunistic fungi such as

320 *Rhizopus microsporus* and *Aspergillus parasiticus*, both parameters were

321 analyzed in inoculated stems. Exposure to *C. heterostrophus*, *F. verticillioides*, *R.*

322 *microsporus*, and *A. parasiticus* all resulted in significant induction of both

323 *ZmTps6/11* transcript levels and zealexin A1, which vary in response to different

324 fungi (Fig. 5) (Huffaker et al., 2011). In the same context the 4 fungal species

325 also led to significant accumulation of *ZmTps21* transcripts and β-costic acid

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326 (Fig. 5). Infection with *C. heterostrophus* led to the highest induction of both

327 *ZmTps21* transcripts and β-costic acid in stems, similar to *ZmTps6/11* transcripts

328 and zealexin levels, respectively (Fig. 5). To further consider the natural

329 occurrence of β-selinene derived metabolites in diverse inbreds (McMullen et al.,

330 2009) we analyzed the scutella tissues of 10-d old seedling plants. β-costic acid

331 was detected in all lines harboring Mo17-like *ZmTps21* alleles (Hp301, TX303,

332 Oh43, Oh7B, Ky21, and W22) and was comparatively absent from all inbreds

333 harboring B73-like *Zmtps21* (Ki3, M37W, MS71, M162W, CML247, Ki11,

334 Mo18W) pseudogenes (Fig. 5; Supplemental Fig. S5 and S6). Collectively,

335 theses results support the existence of a single β-selinene synthase in maize

336 responsible for the production of β-costic acid.

337

338 **In vitro and *in vivo* assays support a defensive role for β-costic acid in**

339 **fungal disease resistance.** In effort to assess physiological roles, we quantified

340 β-costic acid present in replications of field-collected roots of B73, Sweet corn

341 (var. Golden Queen), Mo17 and the *ZmTps21* IBM-RIL 0287. On average,

342 sectors of roots containing visible necrosis from each responsive line contained

343 well over 100 μg g^{-1} FW of β-costic acid (Fig. 5). Using this conservative

344 baseline, we then examined the antimicrobial activity of β-costic acid against *F.*

345 *verticillioides, F. graminearum, R. microsporus, A. parasiticus,* and *C.*

346 *heterostrophus* in liquid culture assays. At 100 µg ml^{-1}, β-costic acid completely

347 inhibited the growth of *R. microsporus* and significantly suppressed the growth of

348 all other fungi (Fig. 6; Supplemental Fig. S9). Importantly, β-costic acid

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349 concentrations as low as 25 µg ml^{-1} retained significant inhibitory activity in each

350 case demonstrating that β-costic acid has the potential to function as wide-

351 spectrum antifungal defense at low doses. To estimate *in vivo* roles, mature roots

352 of greenhouse grown B73, Mo17 and 2 predominantly Mo17 IBM-NILs

353 (Supplemental Fig. S9) where damaged and treated with either H_2O or H_2O

354 containing spores of *F. verticillioides* and *F. graminearum* separately. Seven

355 days later, the B73 inbred and the IBM-NIL (m050) harboring a *Zmtps21*

356 pseudogene displayed significantly greater levels of disease as estimated by

357 *Fusarium* DNA levels compared to Mo17 and the functional *ZmTps21* IBM-NIL

358 (m065) (Fig. 6). Collectively, our results are consistent with ZmTps21 pathway

359 products as mediators of antifungal defenses.

360

361 **Discussion**

362   Maize biochemicals either demonstrated or predicted to mediate insect

363 and pathogen defense include diverse volatiles (Degenhardt, 2009; Degenhardt

364 et al., 2009), benzoxazinoids (Frey et al., 2009; Ahmad et al., 2011; Meihls et al.,

365 2013; Handrick et al., 2016), flavonoids and C-linked flavonoid glycosides (Meyer

366 et al., 2007; Balmer et al., 2013; Casas et al., 2016), non-protein amino acids

367 (Yan et al., 2015), oxylipins (Christensen et al., 2015; Borrego and Kolomiets,

368 2016), and non-volatile terpenoids (Schmelz et al., 2014). Among all biosynthetic

369 classes, terpenoids are the most diverse structurally and in demonstrated

370 breadth of ecological interactions mediated (Gershenzon and Dudareva, 2007).

371 At the genome level, plants commonly possess mid-sized terpene synthase gene

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372   families ranging from 14 to 70 members (Chen et al., 2011). More specifically, in

373   maize use of "terpene" as a keyword search in Phytozome

374   (https://phytozome.jgi.doe.gov) currently reveals more than 30 *Tps* gene models.

375   Collective efforts have resulted in the genetic, biochemical and ecological

376   characterization of approximately half of the maize enzymes encoded by *Tps*

377   with product specificity in the production of mono-, sesqui- and diterpenes

378   (Schnee et al., 2002; Kollner et al., 2004; Schnee et al., 2006; Kollner et al.,

379   2008; Degenhardt et al., 2009; Fu et al., 2016; Richter et al., 2016). Maize

380   terpene volatiles are often highly inducible following insect attack and aid in the

381   attraction of diverse natural enemies both above and below ground (Turlings et

382   al., 1990; Rasmann et al., 2005; Degenhardt et al., 2009). Oxygenated non-

383   volatile terpenoids can also accumulate and act as antifungal agents and insect

384   antifeedants (Schmelz et al., 2011). As part of this biochemical complexity, we

385   demonstrate that maize tissues are capable of accumulating both high levels of

386   the sesquiterpene olefin β-selinene and the corresponding non-volatile

387   oxygenated derivative termed β-costic acid. Intriguingly, β-costic acid is produced

388   in diverse aromatic and medicinal plants widely investigated for bioactive agents

389   driving antibiotic and anti-arthropod activities (Rao and Alvarez, 1981; Wu et al.,

390   2006; Katerinopoulos et al., 2011). Despite the widespread occurrence in nature,

391   Tps essential for the specific *in vivo* production of β-costic acid have not been

392   previously demonstrated *in planta*. We currently describe a maize β-selinene

393   synthase, termed ZmTps21, which is required for the inducible accumulation of

394   β-costic acid.

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395   High tissue concentrations of β-costic acid were first detected in mature

396   field-collected roots of both sweet corn and Mo17 but appeared absent the B73

397   inbred. Use of complimentary mapping resources and the induced production of

398   β-costic acid as a qualitative trait demonstrated single a narrow QTL containing a

399   *Tps* gene candidate.

400   To examine the full-length Mo17 *ZmTps21* allele identified, heterologous

401   expression experiments were conducted in *E. coli* and protein extracts incubated

402   with FPP yielded β-selinene as the dominant volatile product. Based on

403   numerous inbred lines and predicted proteins from genome sequences

404   (Supplemental Fig. S5 and S6), the *in vitro* products of functional ZmTps21 are

405   consistent with the endogenous presence of β-costic acid in all Mo17-like

406   *ZmTps21* lines and likewise an absence in all B73-like *Zmtps21* lines (Fig. 5).

407   Precursors of dominant biochemical defense pathways are commonly the

408   products of fully functional duplicate genes (Kollner et al., 2008; McMullen et al.,

409   2009); however, mGWAS mapping results (Fig. 2) and the exclusive presence of

410   β-costic acid (Fig. 5) in lines with full-length *ZmTps21* alleles collectively support

411   the existence of a single maize β-selinene synthase. At the enzymatic level, the

412   existence of a product specific β-selinene synthase was first reported in 1992

413   through the examination of *Citronella mitis* fruits; however, the specific Tps gene

414   responsible remains unknown (Belingheri et al., 1992). Acid-induced cyclization

415   of germacrenes can also yield selinenes making it highly probable that a

416   germacrene A synthase is responsible for the costol, costal, and costic acid

417   eudesmanes in costus root oil; however, it remains unknown if costus contains a

18

418 specific β-selinene synthase (de Kraker et al., 2001). Further elucidation of the β-

419 costic acid pathway will require the discovery of a yet unresolved cytochrome

420 P450 monooxygenase(s) performing sequential oxidations leading to the

421 carboxylic acid. Characterized germacrene A oxidases from the Asteraceae drive

422 the biosynthesis of germacrene A acid which following acid-induced

423 rearrangement can yield blends that include β-costic acid (Nguyen et al., 2010).

424 A related P450 that directly oxidizes β-selinene to yield β-costic acid is predicted

425 to occur in maize yet remains unknown.

426   While numerous plants in nature constitutively make β-selinene in specific

427 tissues and life stages, β-selinene is rarely detected in maize and has occurred

428 only in the context of pathogen attack (Becker et al., 2014; Sowbhagya, 2014).

429 Consistent with this observation we find *ZmTps21* transcripts largely

430 undetectable in healthy control tissues or those experiencing simple mechanical

431 damage (Fig. 5). In contrast, heat-killed *Fusarium* hyphae and a wide range of

432 live fungal species elicit *ZmTps21* transcript accumulation and β-costic acid

433 production. With conceptual similarities to the zealexin pathway, the elicitation

434 kinetics of both *ZmTps21* transcripts and β-costic acid differ and are temporally

435 behind those of *ZmTps6/11* and zealexins. Given the broader range of fungi

436 species displaying β-costic acid mediated growth suppression at 25 μg ml^{-1}

437 compared to similar assays using zealexins (Huffaker et al., 2011), it is possible

438 that the ZmTps21 pathway exists as an additional potent line of defense

439 activated sequentially as maize plants experience sustained attack. If this

440 hypothesis is true, related studies on maize disease resistance should note

19

441 biological roles for QTLs that include *ZmTps21*. Supportively, independent

442 disease-related QTLs have been detected in broad regions spanning Bin 9.05

443 (Baumgarten et al., 2007; Berger et al., 2014). More specifically *ZmTps21*

444 (GRMZM2G011151) has been identified as uniquely present in transcriptome

445 analyses of resistant inbred lines associated with enhanced antifungal defenses

446 (Lanubile et al., 2014). In an empirical assessment of the *in vivo* role of

447 ZmTps21 derived defenses, root experiments using B73, Mo17 and 2 Mo17 NILs

448 support the suppression of both *F. graminearum* and *F. verticillioides* growth in

449 lines carrying functional Mo17 *ZmTps21* alleles (Fig. 6). Most maize biochemical

450 defenses likely function in the context of complex arrays of bioactive metabolites

451 from numerous pathways. In this context, isogenic mutants in numerous inbred

452 backgrounds would be an ideal and improved platform for the critical examination

453 of *ZmTps21* mediated biological functions. While the present study does not

454 accomplish this long-term goal, we provide a foundation and mechanistic

455 justification for related research directions.

456   Curiously, of lines closely examined at the gene level, β-costic acid

457 biosynthesis mediated by *ZmTps21* is associated with inbreds originating from

458 U.S. breeding programs. In contrast, β-costic acid biosynthetic capacity is largely

459 absent from more geographically diverse accessions. It is tempting to speculate

460 that while the β-costic acid pathway is commonly absent due to a partial gene

461 deletion, *ZmTps21* may have been maintained by positive selection during the

462 breeding of U.S. maize lines. Western corn rootworm (WCR: *Diabrotica virgifera*

463 *virgifera*) larvae exist as a candidate pest pressure known to devastate the roots

20

464 of temperate maize through below-ground herbivory and the promotion of

465 secondary disease (Flint-Garcia et al., 2009; Gray et al., 2009). In growth

466 chamber experiments, maize plants containing a functional *ZmTps21* allele

467 produced both β-selinene and β-costic acid following damage by WCR larvae

468 (Fig. 2). Consistent with a long-term association, unlike the generalist *D. balteata*,

469 WCR larvae were not significantly affected in preference or performance by β-

470 costic acid as a direct defense (Fig. 2). In this context, β-costic acid is likely to be

471 more important in limiting the secondary spread of fungal pathogens promoted by

472 root herbivory. However, while not specifically examined here, we speculate that

473 root pools of β-selinene may serve as a volatile attractant to natural enemies of

474 *Diabrotica* larvae such as entomopathogenic nematodes (Rasmann et al., 2005;

475 Degenhardt et al., 2009). This phenomena has been demonstrated in context of

476 trace amounts of maize root caryophyllene elicited following WCR larval

477 herbivory. More broadly, numerous root terpene volatiles can attract both

478 entomopathogenic and phytopathogenic nematodes, a result that highlights

479 complex tradeoffs in the deployment of rhizosphere signals (Ali et al., 2011).

480   In conclusion, our current study identifies the presence of numerous α/β-

481 selinene derived metabolites in maize tissues following biotic stress. In numerous

482 trials using select maize lines, β-selinene and β-costic acid exist as predominant

483 ZmTps21-derived terpenoids produced following fungal elicitation, long-term root

484 herbivory and combined field pressures. Antifungal assays using both *in vitro* and

485 *in vivo* approaches support an antifungal defense role for ZmTps21 pathway

486 products. Root herbivores are likely to be additionally impacted given that β-

21

487   costic acid can reduce the performance of generalists such as *D. balteata* in

488   controlled bioassays. The discovery of further immune-related biochemical traits

489   is certain to continue given the extreme genetic diversity in maize highlighted by

490   over 8000 representative transcript assemblies detectable in diverse germplasm

491   that are absent from B73 (Hirsch et al., 2014). To fill existing voids highlighted by

492   comparative genomics, the combined application of metabolomics, mapping, and

493   *in vitro* biochemistry provides a useful approach to rapidly connect phenotypes

494   with genotypes (Meihls et al., 2013; Handrick et al., 2016; Richter et al., 2016).

495   Our current identification of ZmTps21 as a β-selinene synthase required for β-

496   costic acid production adds to the foundational knowledge of useful maize

497   biochemical pathways that can be intentionally combined for combating complex

498   biotic pressures.

499

500   **MATERIALS AND METHODS**

501   **Plant and Fungal Materials**

502   Seeds of the Intermated B73×Mo17 (IBM) population of recombinant inbred lines

503   (RILs) and the Goodman diversity panel (Flint-Garcia et al., 2005) were kindly

504   provided by Dr. Peter Balint-Kurti (USDA-ARS, Raleigh, NC, USA) and Dr. Georg

505   Jander (Boyce Thompson Institute, Ithaca, NY, USA) (Supplemental Table S2).

506   The IBM-RILs and Goodman diversity panel (replicate 2) were planted at the

507   Biology Field Station located on the University of California San Diego (UCSD)

508   campus in La Jolla, CA, USA, during the summers of 2015 and 2016,

509   respectively. Field challenged roots from B73, Mo17, hybrid sweet corn (*Zea*

22

510   *mays* var. Golden Queen; Southern States Cooperative, Inc. Richmond, VA,

511   USA) and IBM RILs were recovered 70 days after planting, washed, frozen in

512   liquid N_2, ground to a fine powder and ultimately used for genetic mapping.

513   Seeds of indicated B73×Mo17 near-isogenic lines (provided by the Maize

514   Genetic COOP Stock Center, Urbana, IL, USA), landrace inbreds (B73, Ki3,

515   M37W, Ms71, M162W, CML247, Ki11, Mo18W, Hp301, TX303, Oh43, Oh7B,

516   Ky21, Mo17, and W22; National Genetic Resources Program, Germplasm

517   Resources Information Network, Ames, IA) were germinated in MetroMix 200

518   (Sun Gro Horticulture Distribution, Inc.) supplemented with 14-14-14 Osmocote

519   (Scotts Miracle-Gro) and grown in a greenhouse as previously described

520   (Schmelz et al., 2009) (Supplemental Table S2). Fungal stock cultures of

521   *Rhizopus microsporus* (Northern Regional Research Laboratory stock no.

522   54029), *Fusarium verticillioides* (Northern Regional Research Laboratory; NRRL

523   stock no. 7415), *Fusarium graminearum* (NRRL stock no. 31084) *Aspergillus*

524   *parasiticus* (nor-1), and *Cochliobolus heterostrophus* were grown on V8 agar for

525   12 days before the quantification and use of spores (Huffaker et al., 2011;

526   Huffaker et al., 2013). Heat-killed *Fusarium venenatum* (strain PTA-2684)

527   hyphae was commercially obtained (Monde Nissin Corporation Co.) and used

528   safely for large-scale field mGWAS trials as a non-infectious elicitor lacking

529   known *Fusarium* mycotoxins.

530
531
532   **Genetic Mapping of *ZmTps21***

533 Using the presence of β-costic acid in necrotic tissues as a trait, the B73

534 *Zmtps21* locus was mapped using 216 IBM RIL lines (Lee et al., 2002) and

535 further supported using select B73×Mo17 NILs (Eichten et al., 2011). Marker

536 data for the IBM RIL population were provided by Dr. Peter Balint-Kurti (USDA-

537 ARS, Raleigh, NC). Windows QTL Cartographer (Version 2.5;

538 http://statgen.ncsu.edu/~shchwang/WQTLCart.htm) was employed for metabolite

539 Quantitative Trait Locus (mQTL) analysis with composite interval mapping (CIM).

540 The WinQTLCart program was set as following: CIM program module=Model 6:

541 Standard Model, walking speed=1 cM, control marker numbers=5, window

542 size=10 cM, regression method=backward regression. Permutations (500) were

543 run to determine the $P < 0.05$ logarithm (base 10) of odds (LOD) significance

544 threshold (Churchill and Doerge, 1994). A list of RILs and NILs used for mapping

545 in this study are given in (Supplemental Table S2). In effort to confirm and

546 potentially refine the position of the mQTL identified using CIM, association

547 analyses were also conducted on the IBM RILs using the General Linear Model

548 (GLM) in TASSEL 5.0 (Bradbury et al., 2007) and the Unified Mixed Linear Model

549 (MLM) to effectively control for false positives arising from the differential

550 population structure and familial relatedness present in diversity panels (Yu et al.,

551 2006). Unlike diversity panels, differential population structure and familial

552 relatedness are not typically significant features in biparental RIL panels and thus

553 the GLM and MLM models were predicted to generate similar results in the IBM

554 RIL association analyses. Geneotypic data from imputed IBM RIL SNP markers

555 (July 2012 All Zea GBS final build; www.panzea.org) were used for association

24

556   analyses of root β-costic acid levels in the intermated B73 × Mo17 (IBM)

557   population. 173,984 SNP markers with < 20% missing genotypes and minor

558   allele frequency (MAF) > 15% were used.

559   A metabolite based genome-wide association study (mGWAS) was

560   conducted for elicited levels β-costic acid as a trait in the Goodman diversity

561   panel (Flint-Garcia et al., 2005) using the Unified MLM in TASSEL 5.0 (Yu et al.,

562   2006; Bradbury et al., 2007). Final analyses were conducted with the R package

563   GAPIT (Zhang et al., 2010; Lipka et al., 2012), which involves EMMA (executed

564   by R package) and Compressed MLM (CMLM) population parameters previously

565   determined (P3D) to identify genomic regions putatively associated with the trait.

566   GWAS analyses utilized a B73 Version 2 referenced HapMap consisting of

567   246,477 SNPs previously derived from an Illumina 50K array (Cook et al., 2012)

568   and a genotyping by sequencing (GBS) strategy (Elshire et al., 2011) filtering

569   less than 20% missing genotype data with minor allele frequencies (MAF) > 5%

570   (Samayoa et al., 2015; Olukolu et al., 2016). The kinship matrix (K), estimated

571   from 246,477 SNPs was used jointly with population structure (Q) to improve

572   association analysis (VanRaden, 2008). All metabolite data were $\log_2$

573   transformed prior to statistical analysis to improve normality. The Quantile-

574   Quantile plots and Manhattan plots were constructed in the R package qqman

575   (http://cran.r project.org/web/packages/ qqman) (Turner, 2014).

576

577   **Identification and Quantification of Metabolites**

578   Unless otherwise stated, all maize tissue samples were rinsed with water, frozen

579   in liquid N_2, ground to a fine powder in a mortar and stored at -80 C for further

580   analyses. For Vapor Phase Extraction (VPE) based sample preparation, 50 mg

581   aliquots were first weighed, solvent extracted in bead homogenizer, and

582   derivatized using trimethylsilyldiazomethane as previously described (Schmelz et

583   al., 2004; Schmelz et al., 2011). GC-MS analysis was conducted using an Agilent

584   6890 Series gas chromatograph coupled to an Agilent 5973 mass selective

585   detector (interface temp, 250°C; mass temp, 150°C; source temp, 230°C;

586   electron energy, 70 eV). The GC was operated with a DB-35MS column (Agilent,

587   Santa Clara, USA, 30 m x 0.25 mm x 0.25 µm). The sample was introduced as a

588   splitless injection with an initial oven temperature of 45°C. The temperature was

589   held for 2.25 min, then increased to 300°C with a gradient of 20°C min^{-1}, and

590   held at 300°C for 5 min. GC/EI-MS based quantification of β-costic acid was

591   based upon the slope of an external standard curve constructed from β-costic

592   acid (Ark Pharm, #AK168379) spiked into 50 mg aliquots of frozen powdered

593   untreated maize stem tissues which were then processed using VPE (Schmelz et

594   al., 2004). In representative samples analyzed by GC/EI-MS, β-costol was

595   identified based 99% EI match within the Robert P. Adams essential oil MS

596   library (Allured Books). While not previously detected in maize, β-costol is an

597   anticipated an intermediate in samples rich in both β-selinene and β-costic acid.

598   For headspace recovery of ZmTps21 enzyme products by solid phase

599   micro-extraction (SPME), fibers containing100 µm polydimethylsiloxane

600   (SUPELCO, Belafonte, PA, USA) were placed into reaction vials for 60 min

26

601 incubations at 30°C and then introduced into the GC injector for the analyses of

602 the adsorbed reaction products. GC-MS analyses conducted on SPME samples

603 utilized a splitless injection, a DB-5MS column (Agilent, Santa Clara, USA, 30 m

604 x 0.25 mm x 0.25 μm) and an initial oven temperature of 80°C. The temperature

605 was held for 2 min, then increased to 240°C with a gradient of 7°C min^{-1}, and

606 further increased to 300°C with a gradient of 60°C min^{-1} and a hold of 2 min.

607 Precise instrument settings of the Agilent 5973 mass selective detector were

608 identical to those stated above used for plant samples. For GC-MS analysis with

609 a cooler injector, the injector temperature was reduced from 240°C to 150°C.

610   Volatiles emitted from elicited stems and naturally challenged roots of field

611 grown plants were collected by passing purified air over the tissue samples at

612 600 ml min^{-1} and trapped on inert filters containing 50 mg HayeSep® Q (80-100

613 μm mesh) polymer adsorbent (Sigma-Aldrich, St. Louis, MO, USA). Individual

614 samples were then eluted with 150 μl methylene chloride, and analyzed by GC

615 coupled with flame ionization detector (FID) as previously described (Schmelz et

616 al., 2001). β-selinene and related volatiles were quantified by GC-FID using the

617 slope of an external standard curve of (*E*)-β-farnesene. Select samples were

618 analyzed by GC/EI-MS to confirm individual peak identities of representative

619 replicates. This included the comparison of retention times with authentic

620 standards and by comparison of mass spectra with Wiley, National Institute of

621 Standards and Technology and the Adams libraries.

622   To ensure maximal independence of the second GWAS replicate that was

623 grown in the field, analytical conditions utilized LC-MS instead of GC-MS.

27

624   Reacted stem tissues where first ground to a fine powder with liquid N_2 and

625   weighed out in 50 mg aliquots. Tissue samples were sequentially and additively

626   bead homogenized in 1) 100 μl 1-propanol: acetonitrile: formic acid (1:1:0.01), 2)

627   250 μl acetonitrile: ethyl acetate (1:1), and 3) 100 μl of H_2O. Each combined

628   sample consisted of a co-miscible acidified solvent mixture of primarily 1-

629   propanol: acetonitrile: ethyl acetate: water in the approximate proportion of

630   11:39:28:22 which was then centrifuged at 15,000 rpm for 20 min. Approximately

631   150 μl the particulate free supernatant was carefully removed for LC/MS

632   automated sample analyses utilizing 5 μl injections. The LC consisted of an

633   Agilent 1260 Infinitely series HiP Degasser (G4225A), 1260 binary pump

634   (G1312B), and a 1260 autosampler (G1329B). The binary gradient mobile phase

635   consisted of 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in MeOH

636   (solvent B). Analytical samples were chromatographically separated on a Zorbax

637   Eclipse Plus C18 Rapid Resolution HD column (Agilent: 1.8 μm, 2.1 x 50 mm)

638   using a 0.35 ml min^{-1} flow rate. The mobile phase gradient was: 0–2 min, 5% B

639   constant ratio; 3 min, 24% B; 18 min, 98% B, 25 min, 98% B, and 26 min 5% B

640   for column re-equilibration before the next injection. Eluted analytes underwent

641   electrospray ionization (ESI) via an Agilent Jet Stream Source with thermal

642   gradient focusing using the following parameters: nozzle voltage (500 V), N_2

643   nebulizing gas (flow 12 l min^{-1}, 55 psi, 225°C) and sheath gas (350°C, 12 l min^{-1}).

644   The transfer inlet capillary was 3500V and both MS1 and MS2 heaters were at

645   100°C. Negative ionization [M-H]$^{-}$ mode scans (0.1 amu steps, 2.25 cycles s^{-1})

646   from m/z 100 to 1000 were acquired. After considerable unsuccessful attempts to

28

647   optimize parameters required to obtain meaningful daughter ion fragments from

648   β-costic acid, analyses relied exclusively on the native parent [M-H]$^-$ ion m/z 233

649   and stable retention time of 16.65 min separated from established maize

650   zealexins. Quantification utilized an external standard curve of β-costic acid (Ark

651   Pharm, #AK168379, Libertyville, IL, USA) analyzed under identical conditions.

652

653   **Controlled Maize Elicitation Assays**

654   Controlled maize elicitation assays used 30-40 day old greenhouse plants grown

655   in 1-l plastic pots or in the case of the Goodman diversity panel (second

656   replicate) field grown plants. Plants in damage-related treatment groups were slit

657   in the center, spanning both sides of the stem, with a surgical scalpel that was

658   pulled 8–10 cm upward to create a parallel longitudinal incision. The treatment

659   spanned the upper nodes, internodes, and the most basal portion of unexpanded

660   leaves. All fungal spore inoculations (1 × 10^7 ml^{-1}) treatments were performed in

661   100 µl of H$_2$O. For experiments involving stem elicitation with heat-killed

662   *Fusarium* hyphae, crude material was homogenized in a Waring blender at

663   maximum speed for 3 min in the presence of additional H$_2$O at 20-30% (WT/WT)

664   to create a thick smooth paste. Approximately 500 µl of crude elicitor was

665   introduced into each slit stem followed by sealing the site with clear plastic

666   packing tape to minimize desiccation of the treated stem tissues. For each

667   individual experiment details relating to specific tissues, biological replications

668   and harvest time points are noted in the figures and captions.

669   For the assay of plant responses to long-term western corn rootworm

670   (WCR; *Diabrotica virgifera virgifera*) herbivory, seeds of an IBM line carrying a

671   functional *ZmTps21* gene (IBM-RIL-0287) were grown in 946 ml DM32R cups

672   (Dart Container Corporation, Mason, MI, USA) filled with greenhouse potting

673   media and fertilized following Gassmann et al. (Gassmann et al., 2011). Seeds

674   were planted 1 month prior to WCR inoculation and maintained at 23°C – 28°C in

675   a greenhouse with supplemental daylight balanced illumination on a 16:8 (L:D)

676   photoperiod. Plants were watered daily as needed to prevent saturated soil

677   conditions. Inoculation and care of ≥V5-V6 stage (Abendroth, 2011) treated

678   plants followed from Gassmann et al. (Gassmann et al., 2011). Cups were

679   inoculated with n=10 neonate WCR larvae (obtained from USDA-ARS-NCARL;

680   Brookings, SD, USA) and held in an incubator at 24°C with 40%-60% RH and

681   watered sparingly as needed to minimize pot flooding. The experiment utilized 4

682   replicates per treatment. After 17 d, 1 g samples of insect attacked and healthy

683   root tissues were collected from the plants, frozen on dry ice and stored for

684   chemical analyses.

685

686   ***Diabrotica* Species Preference and Performance Assays**

687   For studies on preference and performance, WCR eggs and *Diabrotica*

688   *balteata* (LeConte) eggs were obtained from USDA-ARS-NCARL (Brookings,

689   SD, USA) and Syngenta (Syngenta Crop Protection AG, CHE), respectively. All

690   larvae were reared on the roots of germinating maize seedlings until use. For

691   both *Diabrotica* species, 3rd instar larvae were used for all experiments. The

692 performance of *D. virgifera* and *D. balteata* larvae was evaluated by placing one

693 pre-weighed larva into individual solo cups (Bioserv, Newark, DE, USA)

694 containing moist filter paper and a 60 mg crown root section from the B73 inbred.

695 Crown roots were covered with 50 µL β-costic acid in EtOH: H_2O (15 %: 85 %) to

696 create a final tissue concentration of 100 µg g^{-1} FW. Control roots were similarly

697 treated with 50 µL EtOH: H_2O (15 %: 85 %). Larval growth was determined after

698 48 h. The preference of the root herbivores given a choice between control and

699 β-costic acid complemented roots was evaluated in 9 cm dia. petri dishes

700 (Greiner Bio-One GmbH, Frickenhausen, DE). Root tissue treatments followed

701 from the performance experiment. One root of each treatment was placed in the

702 petri dishes. Five larvae were introduced in between the two root sections and

703 larvae feeding behavior was recorded at 0.5, 1, 2, 3 and 4 h after start of the

704 trials.

705

706 **RNA Isolation and qRT-PCR**

707 Total RNA was isolated with TRIzol (Invitrogen, Waltham, MA, USA) according to

708 the manufacturer's protocol. First-strand cDNA was synthesized with the

709 RETROscript reverse transcriptase kit (Ambion, Waltham, MA, USA) using

710 random decamer primers. qRT-PCR was performed using Power SYBR Green

711 Master mix (Applied Biosystems, Waltham, MA, USA), and 250 nM primers on a

712 Bio-Rad CFX96^{TM} Real-Time PCR Detection System. Mean cycle threshold

713 values of triplicate reactions were normalized to EF-1α (GenBank accession no.

714 AF136829) (Huffaker et al., 2011). Fold-change calculations were performed

31

715 using the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). qRT-PCR primers used

716 in the current study are listed (Supplemental Table S1)

717
718 **Isolation of *ZmTps21* cDNA from Mo17**
719
720 Total RNA was isolated as described above and subjected to TURBO™ DNA-

721 free™ treatment (Ambion) followed by total RNA purification with RNeasy® Mini

722 protocol for RNA cleanup (Qiagen, Hilden, GR). Approximately 1 µg of an equally

723 mixed RNA pool from Mo17 meristem tissues elicited with heat killed *Fusarium*

724 hyphae collected at different time points (8, 24, 32, and 48 h) was used for the

725 construction of 5'- or 3'-RACE cDNA library with SMARTer RACE 5'/3' Kit

726 (Clontech, Mountain View, CA, USA) in accordance with the manufacturers'

727 protocol. The 5'-end of B73-*Zmtps21* was used to design primers for PCR

728 amplification of the Mo17 *ZmTps21* gDNA. A DNA fragment, which was larger

729 than the one from B73 on the agarose gel, was amplified using primers 5'-

730 TGTGAACCAACAAAGCAAGGC-3' and 5'-GAGCTCACCAATCATAGCCTC-3'

731 cloned and sequenced. Based on the conserved sequences between B73 and

732 Mo17, primers were designed to amplify of the 3' and 5' ends *via* rapid

733 amplification of cDNA ends (RACE) (Clontech) from 5'/3' cDNA libraries of

734 *Fusarium* elicited meristems of Mo17. The complete cDNA sequence of the

735 Mo17 functional *ZmTps21* was amplified with the primers Mo17 *ZmTPS21*F (5'-

736 ATGGATGGTGATATTGCTGCCG-3') and Mo17 *ZmTps21*R (5'-

737 TCAGGCACACGGCTTGAGG-3') from the Mo17 5'-RACE cDNA library. Primers

738 used to amplify *ZmTps21* genomic DNA from B73, W22, CML247 and other

739 diverse inbred lines (Ki3, M37W, MS71, M162W, Ki11, Mo18W, HP301, TX303,

32

740 OH43, Oh7B, KY21, Mo17) are listed (Supplemental Table S1). Corresponding

741 unpublished sequences were deposited in GenBank with following accession

742 numbers (MF614104, MF614105, MF614106, MF614107, MF614108,

743 MF614109, MF614110, MF614111, MF614112, MF614113, MF614114,

744 MF614115).

745

746 **Assay for Terpene Synthase Activity**

747 The complete open reading frame of Mo17 *ZmTps21* was amplified with the

748 primers Mo17 *ZmTps2*1-fwd (CACCATGGATGGTGATATTGCTGCCG) and

749 Mo17 *ZmTps2*1-rev (TCAGGCACACGGCTTGAGGAAC) and the resulting PCR

750 fragment was cloned into the vector pET100/D-TOPO® (Invitrogen, Carlsbad,

751 CA, USA). Sequencing of several clones revealed intact Mo17 *ZmTps2*1 and two

752 cloning artifacts with altered 3' ends. For heterologous expression in *E. coli*, the

753 plasmids were introduced into the strain BL21 Codon Plus (Invitrogen, Carlsbad,

754 CA, USA). Expression was induced by addition of isopropyl-1-thio-D-

755 galactopyranoside to a final concentration of 1 mM. The cells were collected by

756 centrifugation at 4,000g for 6 min, and disrupted by a 4 × 30 sec treatment with a

757 sonicator in chilled extraction buffer (50 mM MOPS, pH 7.0, with 5 mM $MgCl_2$, 5

758 mM sodium ascorbate, 0.5 mM PMSF, 5 mM dithiothreitol and 10% v/v glycerol).

759 The cell fragments were removed by centrifugation at 14,000 g, and the

760 supernatant was desalted into assay buffer (10 mM MOPS, pH 7.0, 1 mM

761 dithiothreitol, 10% v/v glycerol) by passage through a Econopac 10DG column

762 (BioRad, Hercules, CA, USA). Enzyme assays were performed in a Teflon®-

33

763   sealed, screw-capped 1 ml GC glass vial containing 50 µl of the bacterial extract

764   and 50 µl assay buffer with 10 µM (*E,E*)-FPP and 10 mM MgCl$_2$. SPME fiber

765   sample enrichment of adsorbed reaction products and analyses by GC/MS is

766   detailed above in "Identification and Quantification of Metabolites".

767

768   **Bioassays of *in vitro* and *in vivo* β-costic acid activity as an antifungal**

769   **agent**

770   Maize antifungal assays using purified β-costic acid (Ark Pharm, #AK168379)

771   were performed using the Clinical and Laboratory Standards Institute M38-A2

772   guidelines as previously detailed (Schmelz et al., 2011). In brief, a 96-well

773   microtiter plate-based method using a Synergy4 (BioTech Instruments, Inc.)

774   reader was used to monitor fungal growth at 30 °C in broth media through

775   periodic measurements of changes in OD at 600 nm. Each well contained 200 µl

776   of initial fungal inoculum (2.5×10^4 conidia ml^{-1}) with 0.5 µl of either pure DMSO

777   or DMSO containing dilutions of β-costic acid.

778

779   For the mature root infection assays with *Fusarium* pathogens, individual

780   maize plants were greenhouse grown in separate 10-liter pots and supplemented

781   with 14-14-14 Osmocote (Scotts Miracle-Gro) fertilizer. In effort to closely parallel

782   our observations from mature field roots and minimize the invasiveness of

783   belowground treatments, we limited our selection to large nodal roots (≥2mm

784   dia.) containing 1st order lateral roots that were visually apparent and easily

785   accessed following the temporary removal of the pot. Spanning a length of 8 cm,

34

786   at 1 cm intervals selected nodal roots were punctured with a blunt ended circular

787   steel pin (0.6mm dia) creating a total of 9 punctures. Divided across the 9 wound

788   sites per nodal root and depending on treatment, 100 μls of either H_2O or 1×10^7

789   conida mL^{-1} of either *F. verticillioides* (*F.v.*) or *F. graminearum* (*F.g.*) were

790   applied. Treatments were limited to exposed roots growing along the outer-edge

791   of the soil in close contact with the vertical wall of the plastic pot. Following

792   treatments, plants were carefully placed back into the pots for 7 days. For each

793   line grown, namely B73, m050, Mo17 and m065, 3 treatments and 4 replicates

794   were performed (4*4*3 = 48 plants). For determination of the fungal biomass,

795   inoculated and damaged roots were collected 7 days after fungal inoculation.

796   Total genomic DNA was extracted from the infected roots and subjected to real

797   time qPCR using the *F. graminearum*-specific primers for a deoxynivalenol

798   mycotoxin biosynthetic gene (*FgTri6*) and *F. verticillioides* specific primers for a

799   calmodulin (*FvVER1*) gene (Mule et al., 2004; Horevaj et al., 2011) (*SI Appendix*,

800   Table S1). The amount of pathogen DNA relative to plant DNA was estimated by

801   qRT-PCR. Plant DNA quantification utilized a conserved genomic sequence of

802   *ZmTps21/Zmtps21* DNA shared between B73 and Mo17 using forward (gTps21-

803   F, GCAGATGTGTTCGACAAGTTCC) and reverse (gTps21 R-

804   TTACCTGCAGATTTCTCTAAGCTCTC) primers with calculated amplification

805   efficiencies of 102.65-102.89% between inbreds (Supplemental Table S1).

806   Relative amounts of fungal DNA were calculated by the $2^{-\Delta\Delta Ct}$ method,

807   normalized to a conserved genomic sequence of *ZmTps21/Zmtps21* DNA shared

808   between B73 and Mo17.

Statistical Analyses

ANOVAs were performed on the quantified levels of terpenoids, qRT-PCR

transcripts, fungal growth and levels of fungal DNA. Treatment effects were

investigated when the main effects of the ANOVAs were significant (P<0.05).

Tukey tests were used to correct for multiple comparisons between control and

treatment groups. The short-term preference and 2-d performance of *Diabrotica*

larvae on roots, with and without additional β-costic acid, were analyzed with one

sample t-tests and two-way ANOVA using SigmaPlot 13.0 (Systat Software Inc,

San Jose, CA, USA), respectively.

Supplemental Materials

Figure S1. α/β-selinene derived oxidative products, β-costol, β-costal, α-costic

acid and β-costic acid coexist as a network of maize metabolites.

Figure S2. Replicated and comparative association analyses confirm detection

of *ZmTps21* as a gene candidate involved in β-costic acid biosynthesis.

Figure S3. Confirmation of the locus identified by combined linkage and

association mapping based on β-costic acid levels using B73 and Mo17 near

isogenic lines (NILs).

Figure S4. Sequence comparison of Mo17 ZmTps21 with other plant terpene

synthases known to catalyze the protonation of neutral reaction intermediates.

Fig. S5. *ZmTps21* gene structure and sequence polymorphisms across

numerous diverse inbred lines support the occurrence of a common and

conserved B73-like mutation.

36

833   **Figure S6.** Deduced amino acid sequence comparison of ZmTps21 across select

834   maize inbred lines.

835   **Figure. S7.** C-terminal modifications in Mo17 ZmTps21 support an influential role

836   in the protonation of germacrene A as putative reaction intermediate

837   **Figure S8.** Germacrene A is minor yet detectable product of Mo17

838   ZmTps21 and is converted to β-elemene during GC injection at 240°C.

839   **Figure S9.** ZmTps21 derived products inhibit fungal growth at

840   physiologically relevant concentrations *in vitro* and can be assessed *in vivo*

841   using IBM near isogenic lines (NILs).

842   **Table S1.** Primers used for qRT-PCR analysis and sequencing *ZmTps21*

843   genomic DNA

844   **Table S2.** Maize lines specifically used to identify *ZmTps21*

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847   **ACKNOWLEDGMENTS**

Figure Legends

865

866 **Figure 1. β-selinene and β-costic acid can occur as major components of**
867 **maize roots in field grown plants.** Visibly (*A*) infected or (*B*) healthy field
868 collected sweet corn (var. Golden Queen) root samples following
869 trimethylsilyldiazomethane derivatization of carboxylic acids to corresponding
870 methyl esters. Labeled peaks in representative GC/EI-MS total ion
871 chromatograms (TIC) include: 1, β-selinene; 2, α-selinene (shoulder); 3, β-costic
872 acid; 4, zealexin A1; and 5, zealexin B1. The presence of common fatty acids,
873 namely palmitic acid and steric acid, are unchanged in healthy root tissues and
874 directly labeled for reference. Corresponding EI spectra (*m/z*) of (*C*) β-selinene,
875 (*D*) α-selinene, and (*E*) β-costic acid methyl ester from maize field collected
876 roots. (*F*) Proposed α/β-costic acid biosynthetic pathway in maize starting from
877 farnesyl diphosphate (FPP).

878

879 **Figure 2. β-selinene can exist as a dominant elicited volatile and the**
880 **pathway product β-costic acid can reduce herbivore performance.**
881 Representative GC-FID traces of volatile emissions collected from live roots of
882 field grown maize lines (*A*) B73, (*B*) Mo17 and (*C*) IBM-RIL 0287 20 days after
883 pollination. (*D*) Average (*n* = 4, ± SEM) quantity ($\mu g\ 12\ h^{-1}\ g^{-1}$ DW) of β-selinene
884 volatiles emitted from respective maize roots. Representative GC-FID traces of
885 emitted volatiles collected from living (*E*) control B73, (*F*) *C. heterostrophus*-
886 infected B73, (*G*) control Mo17 and (*H*) Mo17 *C. heterostrophus*-infected stems.
887 (*I*) Average (*n* = 4, ± SEM) quantity ($ng\ cm^{-2}\ h^{-1}$) of β-selinene emitted as a
888 volatile from the stems of 5-week-old plants following damage and treatment with
889 H_2O (Dam) or with 100 μl of 1 x10^7 spores *C. heterostrophus (C.h.)*. Within plots
890 *D* and *I*, different letters (a–c) represent significant differences (All ANOVA *Ps* <
891 0.05; Tukey test corrections for multiple comparisons: *P* < 0.05). (*J*) Average (*n* =
892 4, ± SEM) root tissue concentrations ($\mu g\ g^{-1}$ FW) of β-selinene and β-costic acid
893 levels in the roots of IBM-RIL-0287 following 17 days of either no treatment (Ctr)
894 or herbivory by western corn rootworm (WCR) (*Diabrotica virgifera virgifera*)
895 larvae (Student's t-test; one-tailed distribution, equal variance). (*K*) Average
896 WCR (*n* = 18, ± SEM) and *Diabrotica balteata* (*n* = 57, ± SEM) preference over 4
897 h for excised maize roots treated with either EtOH:H_2O (15:85) alone (Control) or
898 the same solution containing β-costic acid to achieve a root tissue concentration
899 of 100 $\mu g\ g^{-1}$ FW. Each replicate (*n)* consisted of assays with 5 individual 3rd
900 instar larvae where distributions were measured at 30,60, 90, 120, 180, 240 min
901 and collectively averaged (one sample t-test, *Ps* > 0.05). (*L*) Average (*n* ≥ 5, ±
902 SEM) performance (% relative weight gain) of 3rd instar WCR and *D. balteata*
903 larvae over 2 days of feeding on root tissues with (+) and without (-) additions of
904 β-costic acid as described in the preference study (two-way ANOVA *P* < 0.05).

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Figure 3. Combined linkage and association mapping identifies ZmTps21 as a candidate β-selinene synthase. (*A*) Major mQTL for β-costic acid production detected on chromosome 9 by composite interval mapping (CIM) using IBM recombinant inbred lines (RILs). (*A-insert*) Comparative association analysis of the IBM-RIL β-costic acid levels using the General Linear Model (GLM) and 173,984 SNPs. The most statistically significant SNP is located at 127,854,265 on Chromosome 9 (B73 RefGen_v2) with a dashed line denoting the 5% Bonferroni correction. (*B*) Quantile-quantile plot for association analysis of β-costic acid levels in the Goodman diversity panel. (*C*) Manhattan plot of the association analysis (MLM) of β-costic acid levels in replicate 1 of the Goodman diversity panel following 3 days of fungal elicitation. Dashed line denotes the 5% bonferroni-corrected threshold for 246,477 SNP markers with the most statistically significant SNP located at 127,858,963 (B73 RefGen_v2) on Chromosome 9. (*D*) Location of the candidate gene *ZmTps21* on the physical map supported by both linkage analysis and association analysis. (*E*) Fine-mapping with IBM near-isogenic lines (NILs); B73 and Mo17 chromosomal segments are represented by blue and red, respectively. β-costic acid chemotypes of IBM-NILs are indicated as GC/EI-MS traces (m/z = 233). (F) Agarose gel PCR amplified products demonstrate a cDNA length polymorphism between B73 *Zmtps21* and Mo17 *ZmTps21* candidates. (*G*) Diagrammatic structures of B73 *Zmtps21* and Mo17 *ZmTps21* genes based on sequencing. Exons and introns are denoted as rectangular bars and as black lines, respectively. Open rectangle indicates the missing B73 genomic DNA and relative position of encoded conserved RXR and DDXXD motifs terpene cyclase activity.

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Figure 4. Mo17 *ZmTps21* encodes a functional β-selinene synthase. (*A*) Mo17 ZmTps21 was in *Escherichia coli* and the resulting protein extract was incubated with (*E,E*)-farnesyl diphosphate (FPP). Mo17 ZmTps21 products were collected using solid-phase microextraction and analyzed by GC/MS revealing (2) β-selinene as the dominant product with lower yet detectable levels of (1) β-elemene (germacrene A rearrangement product) and (3) α-selinene. (*B*) Celery fruit essential oil was used as a natural product standard for β-selinene/α-selinene (9:1).

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Figure 5. Zm*Tps21* transcripts are elicited by diverse fungi and precede β-costic acid accumulation detectable in diverse maize lines. Average (n = 4; ±SEM) Mo17 (*A*) *ZmTps21* (*B*) β-costic acid (*C*), *ZmTps6/11* and (*D*) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations (μg g^{-1} FW) in intact control stems (Con) or those damaged and treated with either H$_2$0 (Dam), or a heat-killed *Fusarium* elicitor (F.E.) hyphae preparation after 1, 2, or 4 days. Average (n = 4; ±SEM) Mo17 (*E*) *ZmTps21* (*F*) β-costic acid (*G*), *ZmTps6/11* and (*H*) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations (μg g^{-1} FW) in intact control stems (Con) or those damaged and treated with either 100 μl of H$_2$0 (Dam) alone or spore suspensions (1×10^7 ml^{-1}) of *R. microsporus* (*R.m.*), *A. parasiticus* nor-1 (*A.p.*), *F. verticillioides* (*F.v.*), or *C. heterostrophus* (*C.h.*) and harvested at 2 and 4 days for transcripts and metabolites, respectively. (*I*) Average (n = 4, ± SEM) β-costic acid concentrations (μg g^{-1} FW) in the scutella of 10-d-old maize seedlings from 15 inbred maize lines and mature field collected roots displaying necrosis. Hybrids include sweet corn (var. Golden Queen; GQ) and IBM-RIL0287. Within plots, different letters (a-e) represent significant differences (all ANOVA P < 0.05; Tukey test corrections for multiple comparisons: P < 0.05).

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Figure 6. ZmTps21 derived products inhibit *Fusarium* fungi *in vitro* and correspond with improved disease resistance *in vivo*. Average (n = 8, ± SEM) fungal growth estimates (600 nm OD) of (*A*) *F. verticillioides*, and (*B*) *F. graminearum*, in liquid media in the presence of β-costic acid at 0 (○), 25 (●), and 100 (△) µg ml^{-1}. Average (n = 4, ± SEM) ratio of fungal DNA / plant DNA levels present in maize roots 7 days after damage and inoculation with 100 µls of either H$_2$O or 1 × 10^7 conida mL^{-1} of (*C*) *F. verticillioides* and *(D) F. graminearum* in B73, Mo17 and IBM-NILs harboring active (+; m065) and inactive (-; m050) alleles of *ZmTps21*. Within plots, different letters (a–c) represent significant differences (All ANOVA P < 0.05; Tukey test corrections for multiple comparisons: P < 0.05).

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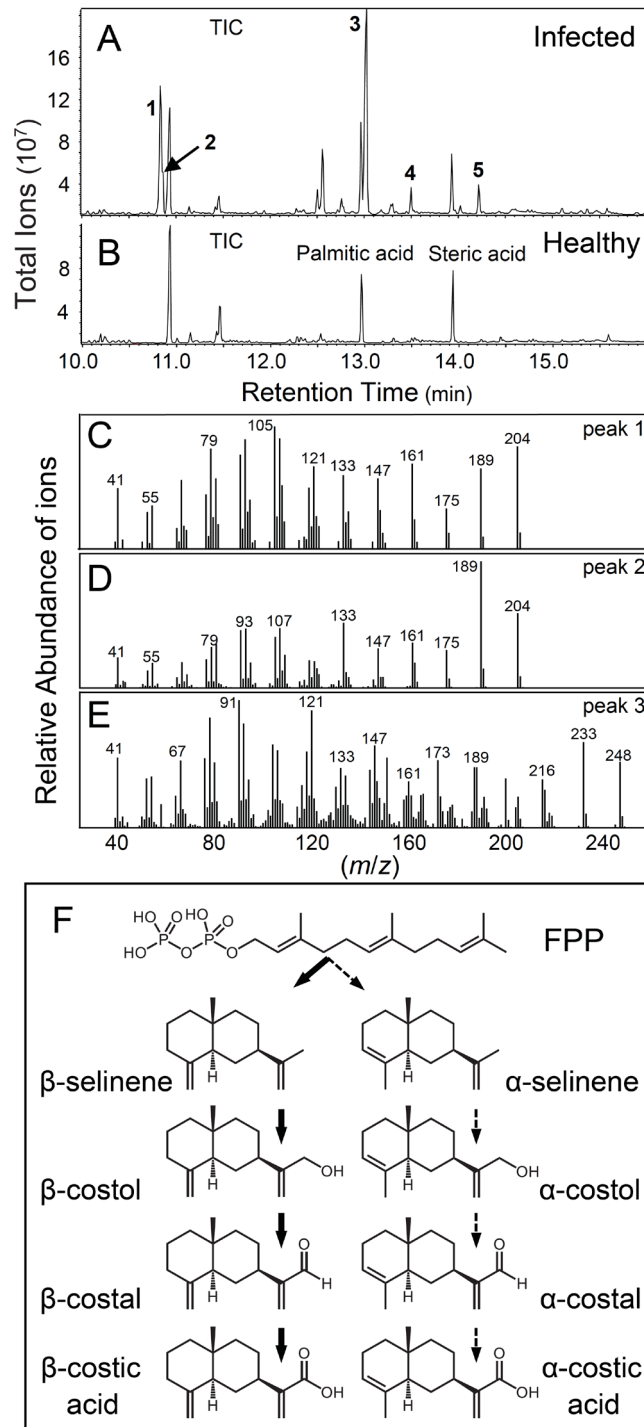1219   association studies. Nat Genet **42:** 355-U118

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Figure 1. β-selinene and β-costic acid can occur as major components of maize roots in field grown plants. Visibly (*A*) infected or (*B*) healthy field collected sweet corn (var. Golden Queen) root samples following trimethylsilyldiazomethane derivatization of carboxylic acids to corresponding methyl esters. Labeled peaks in representative GC/EI-MS total ion chromatograms (TIC) include: 1, β-selinene; 2, α-selinene (shoulder); 3, β-costic acid; 4, zealexin A1; and 5, zealexin B1. The presence of common fatty acids, namely palmitic acid and steric acid, are unchanged in healthy root tissues and directly labeled for reference. Corresponding EI spectra (*m/z*) of (*C*) β-selinene, (*D*) α-selinene, and (*E*) β-costic acid methyl ester from maize field collected roots. (*F*) Proposed a/β-costic acid biosynthetic pathway in maize starting from farnesyl diphosphate (FPP).
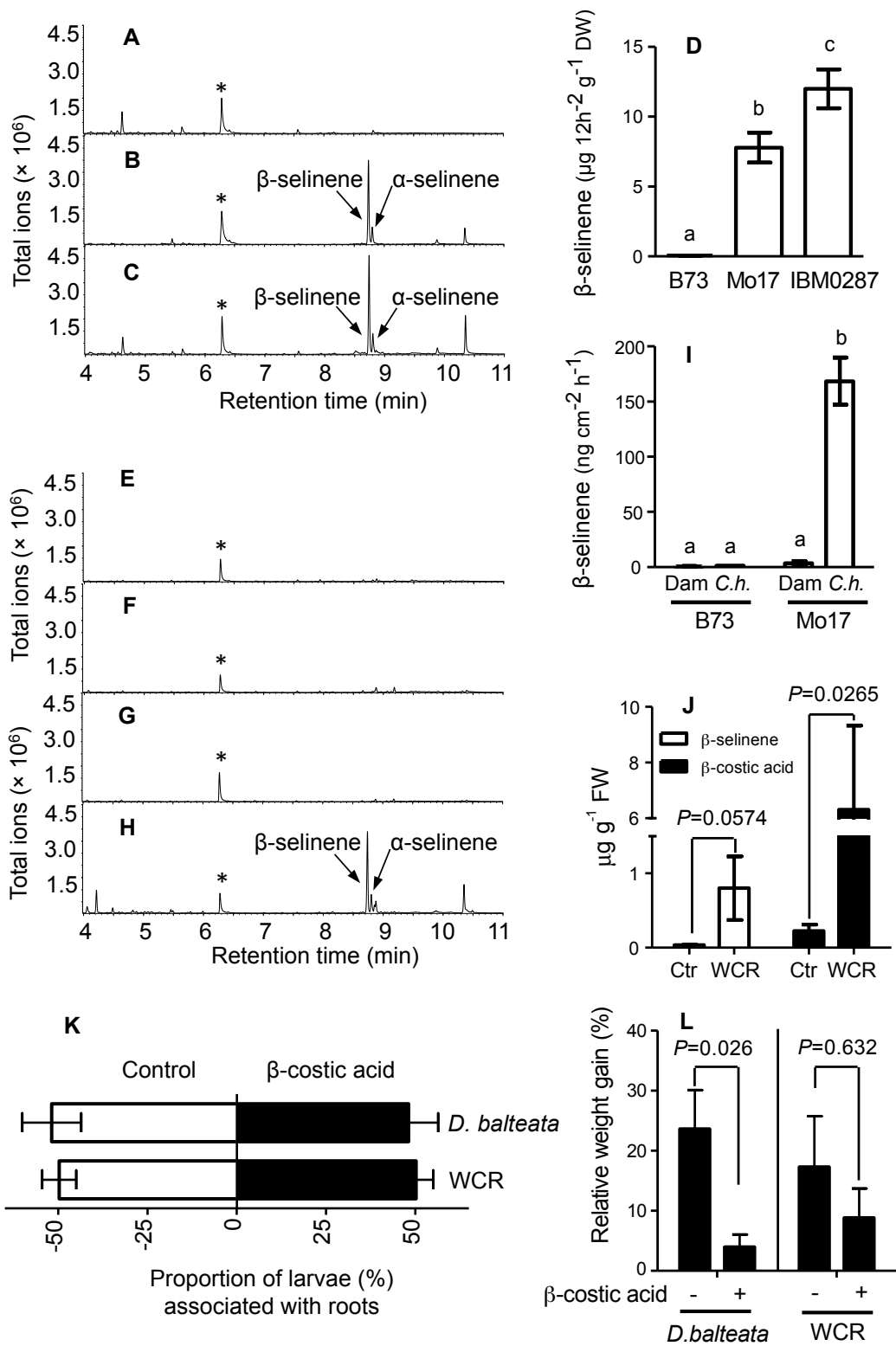
Figure 2. β-selinene can exist as a dominant elicited volatile and the pathway product β-costic acid can reduce herbivore performance. Representative GC-FID traces of volatile emissions collected from live roots of field grown maize lines (*A*) B73, (*B*) Mo17 and (*C*) IBM-RIL 0287 20 days after pollination. (*D*) Average (n = 4, ± SEM) quantity (μg 12 h^{-1} g^{-1} DW) of β-selinene volatiles emitted from respective maize roots. Representative GC-FID traces of emitted volatiles collected from living (*E*) control B73, (*F*) *C. heterostrophus*-infected B73, (*G*) control Mo17 and (*H*) Mo17 *C. heterostrophus*-infected stems. (*I*) Average (n = 4, ± SEM) quantity (ng cm^{-2} h^{-1}) of β-selinene emitted as a volatile from the stems of 5-week-old plants following damage and treatment with H$_2$O (Dam) or with 100 μl of 1 x10^7 spores *C. heterostrophus (C.h.)*. Within plots *D* and *I*, different letters (a–c) represent significant differences (All ANOVA *Ps* < 0.05; Tukey test corrections for multiple comparisons: *P* < 0.05). (*J*) Average (n = 4, ± SEM) root tissue concentrations (μg g^{-1} FW) of β-selinene and β-costic acid levels in the roots of IBM-RIL-0287 following 17 days of either no treatment (Ctr) or herbivory by western corn rootworm (WCR) (*Diabrotica virgifera virgifera*) larvae (Student's t-test; one-tailed distribution, equal variance). (*K*) Average WCR (n = 18, ± SEM) and *Diabrotica balteata* (n = 57, ± SEM) preference over 4 h for excised maize roots treated with either EtOH:H$_2$O (15:85) alone (Control) or the same solution containing β-costic acid to achieve a root tissue concentration of 100 μg g^{-1} FW. Each replicate (n) consisted of assays with 5 individual 3rd instar larvae where distributions were measured at 30,60, 90, 120, 180, 240 min and collectively averaged (one sample t-test, *Ps* > 0.05). (*L*) Average (n ≥ 5, ± SEM) performance (% relative weight gain) of 3rd instar WCR and *D. balteata* larvae over 2 days of feeding on root tissues with (+) and without (-) additions of β-costic acid as described in the preference study (two-way ANOVA *P* < 0.05).
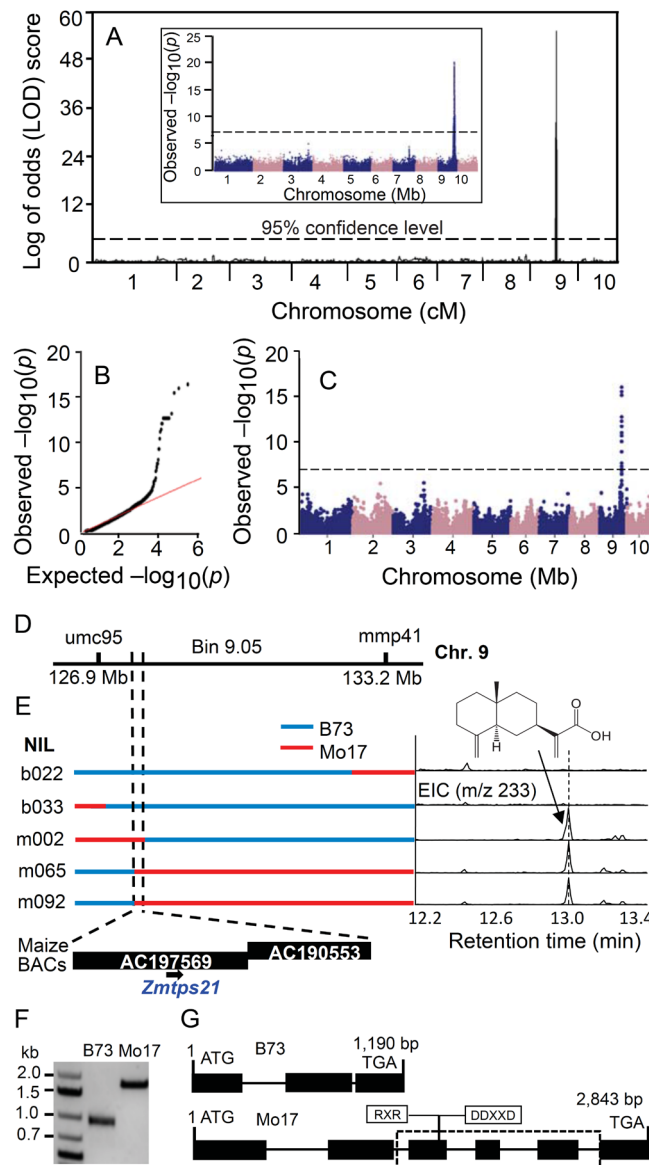
Figure 3. Combined linkage and association mapping identifies ZmTps21 as a candidate β-selinene synthase. (*A*) Major mQTL for β-costic acid production detected on chromosome 9 by composite interval mapping (CIM) using IBM recombinant inbred lines (RILs). (*A-insert*) Comparative association analysis of the IBM-RIL β-costic acid levels using the General Linear Model (GLM) and 173,984 SNPs. The most statistically significant SNP is located at 127,854,265 on Chromosome 9 (B73 RefGen_v2) with a dashed line denoting the 5% Bonferroni correction. (*B*) Quantile-quantile plot for association analysis of β-costic acid levels in the Goodman diversity panel. (*C*) Manhattan plot of the association analysis (MLM) of β-costic acid levels in replicate 1 of the Goodman diversity panel following 3 days of fungal elicitation. Dashed line denotes the 5% bonferroni-corrected threshold for 246,477 SNP markers with the most statistically significant SNP located at 127,858,963 (B73 RefGen_v2) on Chromosome 9. (*D*) Location of the candidate gene *ZmTps21* on the physical map supported by both linkage analysis and association analysis. (*E*) Fine-mapping with IBM near-isogenic lines (NILs); B73 and Mo17 chromosomal segments are represented by blue and red, respectively. β-costic acid chemotypes of IBM-NILs are indicated as GC/EI-MS traces (*m/z* = 233). (F) Agarose gel PCR amplified products demonstrate a cDNA length polymorphism between B73 *Zmtps21* and Mo17 *ZmTps21* candidates. (*G*) Diagrammatic structures of B73 *Zmtps21* and Mo17 *ZmTps21* genes based on sequencing. Exons and introns are denoted as rectangular bars and as black lines, respectively. Open rectangle indicates the missing B73 genomic DNA and relative position of encoded conserved RXR and DDXXD motifs terpene cyclase activity.
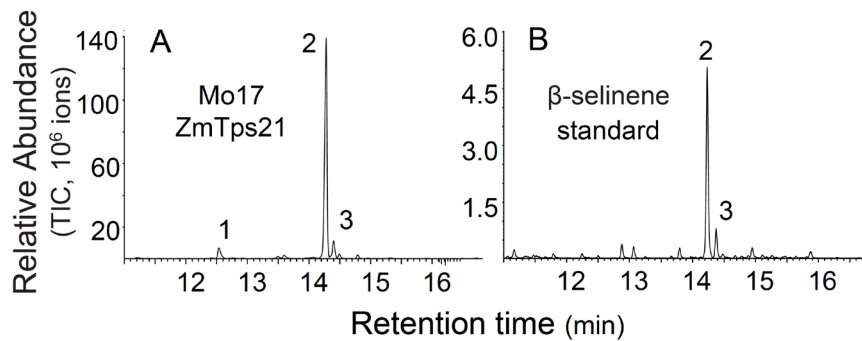
Figure 4. Mo17 *ZmTps21* encodes a functional β-selinene synthase. (*A*) Mo17 ZmTps21 was in *Escherichia coli* and the resulting protein extract was incubated with (*E,E*)-farnesyl diphosphate (FPP). Mo17 ZmTps21 products were collected using solid-phase microextraction and analyzed by GC/MS revealing (2) β-selinene as the dominant product with lower yet detectable levels of (1) β-elemene (germacrene A rearrangent product) and (3) α-selinene. (*B*) Celery fruit essential oil was used as a natural product standard for β-selinene/α-selinene (9:1).

Figure 5. **ZmTps21 transcripts are elicited by diverse fungi and precede β-costic acid accumulation detectable in diverse maize lines**. Average (n = 4; ±SEM) Mo17 (A) *ZmTps21* (B) β-costic acid (C), *ZmTps6/11* and (D) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations (µg g^{-1} FW) in intact control stems (Con) or those damaged and treated with either H$_2$O (Dam), or a heat-killed *Fusarium* elicitor (F.E.) hyphae preparation after 1, 2, or 4 days. Average (n = 4; ±SEM) Mo17 (E) *ZmTps21* (F) β-costic acid (G), *ZmTps6/11* and (H) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations (µg g^{-1} FW) in intact control stems (Con) or those damaged and treated with either 100 µl of H$_2$0 (Dam) alone or spore suspensions (1×107 ml^{-1}) of *R. microsporus* (*R.m.*), *A. parasiticus* nor-1 (*A.p.*), *F. verticillioides* (*F.v.*), or *C. heterostrophus* (*C.h.*) and harvested at 2 and 4 days for transcripts and metabolites, respectively. (I) Average (n = 4, ± SEM) β-costic acid concentrations (µg g^{-1} FW) in the scutella of 10-d-old maize seedlings from 15 inbred maize lines and mature field collected roots displaying necrosis. Hybrids include sweet corn (var. Golden Queen; GQ) and IBM-RIL0287. Within plots, different letters (a-e) represent significant differences (all ANOVA *P* < 0.05; Tukey test corrections for multiple comparisons: *P* < 0.05).
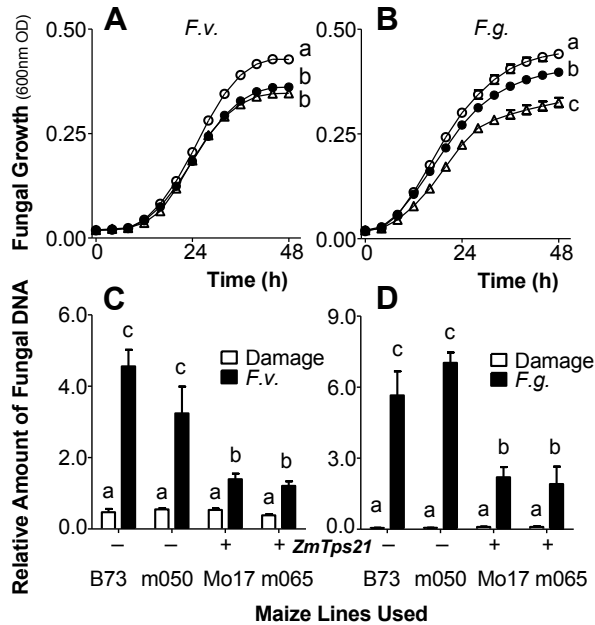
Figure 6. ZmTps21 derived products inhibit *Fusarium* fungi *in vitro* and correspond with improved disease resistance *in vivo*. Average (n = 8, ± SEM) fungal growth estimates (600 nm OD) of (A) *F. verticillioides*, and (B) *F. graminearum*, in liquid media in the presence of β-costic acid at 0 (○), 25 (●), and 100 (△) μg ml^{-1}. Average (n = 4, ± SEM) ratio of fungal DNA / plant DNA levels present in maize roots 7 days after damage and inoculation with 100 μls of either H_2O or 1×10^7 conida ml^{-1} of (C) *F. verticillioides* and (D) *F. graminearum* in B73, Mo17 and IBM-NILs harboring active (+; m065) and inactive (-; m050) alleles of *ZmTps21*. Within plots, different letters (a–c) represent significant differences (All ANOVA *P* < 0.05; Tukey test corrections for multiple comparisons: *P* < 0.05).

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