Running title: Mite adaptation to Arabidopsis

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Rapid specialization of counter defenses enables two-spotted spider mite to adapt to novel plant hosts

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30 **One sentence summary**

31 Mites can evolve efficient detoxification-based adaptations in ≈25 generations of
32 selection on initially unfavourable plant host, demonstrating that specialization to plant
33 resistance traits can occur within the ecological timescale.

34

35 **Author contributions**

36 Investigation: GS, KB, HR, VA, SD, EW, MM and VZ; Writing: GS, KB, VZ, MG and VG;
37 Supervision: VZ, AGC, MG and VG; Conceptualization, Funding Acquisition, Resources:
38 AGC, MG and VG.

39

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Abstract

Genetic adaptation, occurring over a long evolutionary time, enables host-specialized herbivores to develop novel resistance traits and to efficiently counteract the defenses of a narrow range of host plants. In contrast, physiological acclimation, leading to the suppression and/or detoxification of host defenses is hypothesized to enable broad-generalists to shift between plant hosts. However, the host adaptation mechanisms used by generalists composed of host-adapted populations are not known. *Tetranychus urticae* is an extreme generalist herbivore whose individual populations perform well only on a subset of potential hosts. We combined experimental evolution, *Arabidopsis* genetics, mite reverse genetics, and pharmacological approaches to examine mite host adaptation upon the shift of a bean-adapted population to *Arabidopsis thaliana*. We showed that cytochrome P450 monooxygenases are required for mite adaptation to *Arabidopsis*. We identified activities of two tiers of P450s: general xenobiotic-responsive P450s that have a limited contribution to mite adaptation to *Arabidopsis* and adaptation-associated P450s that efficiently counteract *Arabidopsis* defenses. In ≈25 generations of mite selection on *Arabidopsis* plants, mites evolved highly efficient detoxification-based adaptation, characteristic of specialist herbivores. This demonstrates that specialization to plant resistance traits can occur within the ecological timescale, enabling the two-spotted spider mite to shift to novel plant hosts.

Keywords: biochemical adaptation; detoxification; host shift; *NADPH-cytochrome P450 reductase*; plant-herbivore interaction; RNAi; *Tetranychus urticae* Koch (two-spotted spider mite); xenobiotic responsiveness

Introduction

During millions of years of co-evolution with their host plants, herbivores have developed two main strategies to counteract plant resistance traits. Specialist herbivores have evolved highly efficient adaptation mechanisms against a limited set of host defenses, including modified feeding behavior (Helmus and Dussourd, 2005), suppression of plant defenses (Zhao *et al.*, 2015), reduced xenobiotic target site sensitivity (Dobler *et al.*, 2012), sequestration (Beran *et al.*, 2018), and detoxification of plant toxins (Ratzka *et al.*, 2002). In contrast, generalist herbivores evolved an innate ability to feed on a broad range of host plants that display a wide array of resistance traits (Barrett and Heil, 2012; Despres *et al.*, 2007; Heidel-Fischer and Vogel, 2015). The generalist lifestyle is considered to be ecologically and evolutionarily advantageous (Futuyma and Moreno, 1988; Loxdale and Harvey, 2016). However, the fitness trade-offs arising from the biochemical and physiological costs of polyphagy have been proposed to select for the specialization of herbivore–plant interactions (Dall and Cuthill, 1997). Generalist herbivores evolved two main strategies to counteract a diverse array of plant host defenses. Broad-generalists, where all individuals can feed on a wide range of plant hosts, rely on rapid transcriptional plasticity to colonise distantly related plants (Fenton *et al.*, 1998; Mathers *et al.*, 2017). However, the majority of generalist herbivores are regarded as composites of populations that themselves thrive on a subset of potential hosts (Barrett and Heil, 2012; Fox and Morrow, 1981; Peccoud *et al.*, 2009). These populations exist in a wide range of host specializations and are proposed to ultimately lead to the formation of specialist species (Peccoud *et al.*, 2009). Consistent with these theoretical predictions, specialist herbivores dramatically outnumber generalists (Rafter and Walter, 2020). Specialization to plant resistance traits is assumed to occur over a long evolutionary time and evokes complex adaptive genetic changes that result in the establishment of highly efficient adaptation mechanisms against a limited set of host defenses. Despite our understanding of host specialization in specialist herbivores, the mechanisms that mediate the formation of host-adapted populations in generalist herbivores are poorly understood.

98 Attenuation of plant responses induced by herbivore feeding has been proposed
99 to be one of the mechanisms of host adaptation (Kant *et al.*, 2008; Musser *et al.*, 2012;
100 Wu *et al.*, 2012; Zarate *et al.*, 2007). Consistently, effectors that modulate plant
101 defenses have been identified in secretions of a number of generalist herbivores
102 belonging to different feeding guilds (Bass *et al.*, 2013; Basu *et al.*, 2018; Hogenhout and
103 Bos, 2011; Jonckheere *et al.*, 2016; Kaloshian and Walling, 2016; Musser *et al.*, 2002; Wu
104 *et al.*, 2012). Their mode of action is largely unknown but, to be effective against many
105 host plants, it is assumed that they either target conserved compounds or pathways
106 associated with plant defense or have a broad-spectrum activity with only a specific
107 subset being effective against any particular host. Another general mechanism of host
108 adaptation is metabolic resistance whereby herbivores effectively detoxify ingested
109 plant toxins. In specialists, metabolic resistance is based on a limited number of
110 detoxification enzymes that have high specificity and efficiency for a given plant toxin
111 (Gloss *et al.*, 2014; Heidel-Fischer *et al.*, 2019; Li *et al.*, 2003; Mao *et al.*, 2006; Ratzka *et*
112 *al.*, 2002; Wittstock *et al.*, 2004). Genes encoding these enzymes usually carry
113 nucleotide substitutions in coding regions that increase their activity against the plant
114 toxin (Dobler *et al.*, 2012; Gloss *et al.*, 2014; Wen *et al.*, 2006) and/or in the promoter
115 sequences resulting in their constitutive and high level of expression (Bass *et al.*, 2013).
116 In contrast, it is assumed that generalist herbivores rely on ubiquitous classes of
117 detoxification enzymes (e.g. carboxyl/cholinesterases (CCEs), cytochrome P450
118 monooxygenases (CYP450s), glutathione S-transferases (GSTs), UDP-glycosyltransferases
119 (UGTs), and ABC transporters (ABCs)) that were shown to accept structurally diverse
120 substrates which they metabolize with low levels of activity (Halon *et al.*, 2015; Li *et al.*,
121 2004; Shi *et al.*, 2018; Snoeck *et al.*, 2019). Genes encoding general detoxification
122 enzymes have undergone extensive amplification and neofunctionalization, and are
123 transcriptionally responsive to a wide range of xenobiotics (Govind *et al.*, 2010; Grbic *et*
124 *al.*, 2011; Muller *et al.*, 2017; Schweizer *et al.*, 2017; Wybouw *et al.*, 2015; Zhurov *et al.*,
125 2014). Thus, it is hypothesized that attenuation of plant defenses combined with the

126 expanded and functionally versatile detoxification capabilities enable generalist
127 herbivores to cope with diverse allelochemicals and to feed on many host plants.

128 The two-spotted spider mite (TSSM) *Tetranychus urticae* is an extreme generalist
129 herbivore that feeds on over 1,100 plant species from more than 100 families (Migeon
130 and Dorkeld, 2021). Such a wide host range indicates that TSSM can counteract a great
131 diversity of plant resistance traits. However, individual TSSM populations do not
132 perform equally well on all potential host plants (Agrawal *et al.*, 2002; Fellous *et al.*,
133 2014; Fry, 1989). Instead, TSSM has an outstanding ability to adapt to new hosts (Fry,
134 1989; Gould, 1979; Magalhaes *et al.*, 2007; Wybouw *et al.*, 2015). The mechanism of this
135 host adaptability is not known. The analysis of plant transcriptional changes following
136 the host shift revealed that some TSSM populations can suppress plant induced
137 responses (Kant *et al.*, 2008; Wybouw *et al.*, 2015). At the same time, TSSM massively
138 reprograms its detoxification capacity (Dermauw *et al.*, 2013; Wybouw *et al.*, 2015;
139 Zhurov *et al.*, 2014) and the complement of its salivary secretions (Jonckheere *et al.*,
140 2018; Jonckheere *et al.*, 2016; Villarroel *et al.*, 2016). However, no functional evidence
141 explains whether these changes contribute to TSSM host adaptation or if they merely
142 reflect stress responses or different feeding physiology due to the host shift. In addition,
143 it remains unclear if changes to the initial xenobiotic responses are sufficient for TSSM
144 adaptation to a new host, or if additional changes are required for the evolution of
145 TSSM host adaptation.

146 Even though plants belonging to the Brassicaceae family have been recorded as
147 TSSM plant hosts (Migeon and Dorkeld, 2021), *Arabidopsis thaliana* is a non-preferred
148 host for the TSSM London reference population that is reared on a bean – *Phaseolus*
149 *vulgaris* (Zhurov *et al.*, 2014). In an experimental evolutionary setup, we adapted the
150 TSSM London ancestral population to *Arabidopsis.* Using functional pharmacological and
151 reverse genetics experiments that independently suppressed the activities of families of
152 mite detoxification enzymes we provide, for the first time, *in vivo* functional evidence
153 that *T. urticae* requires cytochrome P450 monooxygenase activity for adaptation to
154 *Arabidopsis*. We identified activities of two tiers of P450s: general xenobiotic-responsive

155 P450s that have a limited contribution to mite adaptation to *Arabidopsis* and
156 adaptation-associated P450s that efficiently counteract *Arabidopsis* defenses. Our data
157 reveal that in ≈25 generations, mites established a highly efficient detoxification
158 response to *Arabidopsis* defenses, demonstrating that specialization to plant resistance
159 traits can occur within an ecological timescale.
160
161 **Results**
162 **TSSM feeding induces complex JA-regulated *Arabidopsis* defenses**
163 To understand the mechanism of TSSM adaptation to *Arabidopsis*, we first characterized
164 the complexity of *Arabidopsis* defenses that mites have to overcome in order to use
165 *Arabidopsis* as a host. Jasmonic acid (JA) and its bioactive conjugate jasmonoyl-
166 isoleucine (JA-Ile), Figure 1A, were shown to be required for *Arabidopsis* defenses
167 against TSSM herbivory (Zhurov *et al.*, 2014). A dramatic 5-fold decrease of mite
168 fecundity on methyl jasmonate (MeJA) treated Col-0 plants, Figure 1B, indicates that JA-
169 induced defenses are sufficient for *Arabidopsis* resistance against TSSM. JA
170 responsiveness is mediated by the MYC2, MYC3, and MYC4 (MYC2,3,4) transcriptional
171 activators (Devoto *et al.*, 2005; Fernandez-Calvo *et al.*, 2011), and consistently, *myc2*
172 *myc3 myc4* (*myc2,3,4*) mutant plants are extremely susceptible to TSSM, Figure 1B.
173 Among MYC2,3,4-regulated genes are *CYP79B2* and *CYP79B3* that are required for the
174 synthesis of Trp-derived secondary metabolites (Figure 1A, (Hull *et al.*, 2000; Mikkelsen
175 *et al.*, 2000; Schweizer *et al.*, 2013). One class of these metabolites are indole
176 glucosinolates, shown to protect *Arabidopsis* plants against herbivory of a wide range of
177 arthropods, including mites (Elbaz *et al.*, 2012; Hopkins *et al.*, 2009; Kim and Jander,
178 2007; Kim *et al.*, 2008; Whiteman *et al.*, 2012; Whiteman *et al.*, 2011; Widemann *et al.*,
179 2021; Zhurov *et al.*, 2014). The modest increase in fecundity when TSSM fed on *cyp79b2*
180 *cyp79b3* (*cyp79b2,b3)* plants, Figure 1B, indicates that besides indole glucosinolates,
181 there are other MYC2,3,4-regulated defenses that prominently protect *Arabidopsis*
182 plants against mites. Thus, there are at least two distinct classes of JA-regulated
183 *Arabidopsis* resistance traits against TSSM that are *CYP79B2 CYP79B3*-dependent and -
184 independent.

185

186 **Mites can adapt to a complex array of *Arabidopsis* defenses**

187 To test if TSSM can adapt to the complex array of *Arabidopsis* defenses, we used an
188 experimental evolutionary setup where an ancestral TSSM population that is highly
189 susceptible to *Arabidopsis* defenses (Zhurov *et al.*, 2014) was transferred and
190 continuously maintained on *Arabidopsis* plants. The improvement of mite fitness on a
191 new host has been reported to occur already after 5-10 generations (Agrawal, 2000; Fry,
192 1989; Fry, 1990), however, the improvement is gradual and reaches a stable state after
193 15-20 generations, depending on the ancestral population and the plant host (Agrawal,
194 2000; Fry, 1989; Magalhaes *et al.*, 2009). We have initiated the analysis of mite
195 adaptation after 18 months (≥25 generations) of selection, Figure 2A, to ensure
196 consistency of mite adaptation state throughout the analysis. We initially infested
197 *Arabidopsis* plants with approximately 1,000 fertilized female mites in a triplicated
198 experiment on two *Arabidopsis* genotypes: a) Col-0, fully defended, wild-type plants,
199 and b) *cyp79b2 cyp79b3* mutant plants that lack Trp-derived compounds but display the
200 remaining JA-regulated defenses, Figures 1A and 2A. The performance of the ancestral
201 and selected mite populations was subsequently quantified by counting the total
202 number of eggs, larvae, nymphs, and adults derived from 20 adult females in 7 days. The
203 performance was measured in two experimental regimes: a) direct transfer, where the
204 initial mites were moved from their corresponding rearing plant hosts directly to the
205 experimental plants, and b) indirect transfer, which included mite maintenance on bean
206 plants for two generations before their transfer to the experimental plants. The
207 performance of the ancestral and selected mite populations on bean, or *Arabidopsis*
208 *cyp79b2 cyp79b3* and Col-0 plants had similar patterns in both direct (Supplemental
209 Figure S1) and indirect (Figure 2B) transfer regimes, indicative of genetic adaptation that
210 is independent of maternal and environmental physiological effects.

211 On bean plants, Figure 2B top, *Arabidopsis*-selected mite populations had slightly
212 but significantly lower performance relative to the ancestral population, indicating that
213 TSSMs can evolve the ability to exploit different hosts without a major reduction in their

214 performance on the ancestral plants. On *cyp79b2 cyp79b3* plants, Figure 2B middle, the
215 two *Arabidopsis*-selected mite populations performed significantly better than the
216 ancestral population, suggesting that they can overcome *CYP79B2 CYP79B3*-
217 independent *Arabidopsis* defenses to a similar extent. On Col-0 plants, Figure 2B bottom,
218 only mites selected on Col-0 had increased performance over the ancestral population,
219 showing that Col-0 selected mites were also able to adapt to *CYP79B2 CYP79B3*-
220 dependent defenses. However, mites selected on *cyp79b2 cyp79b3* plants and the
221 ancestral mite population that were not exposed to *CYP79B2 CYP79B3*-dependent
222 *Arabidopsis* defenses were susceptible to these defenses and had similar and low
223 performances when they fed on Col-0 plants.

224 We have arbitrarily chosen populations #3 of *cyp79b2 cyp79b3* and Col-0
225 selected mites for subsequent studies that were all performed in indirect transfer
226 regimes. The analysis of additional mite fitness parameters, fecundity, and *Arabidopsis*
227 leaf damage caused by mite feeding (Figure 2C and D, respectively) confirmed the
228 adaptation status of these mite populations (from now on referred to as *cyp*-a (for #3
229 *cyp79b2 cyp79b3* selected mite population), Col-a (for #3 Col-0 selected mite population)
230 and bean-a (for the ancestral bean-adapted London TSSM population). Thus, our data
231 demonstrate that TSSM can adapt to novel plant hosts that have a complex array of
232 defenses. Importantly, *Arabidopsis*-adapted mite populations retained high fitness on
233 bean plants, thus, they expanded their host range without losing the ability to feed on
234 the ancestral host. Moreover, mite adaptation to *CYP79B2 CYP79B3*-independent
235 *Arabidopsis* defenses (present in both types of *Arabidopsis*-selected mite populations)
236 can be uncoupled from mite adaptation to Trp-derived defenses (present only in mites
237 selected on Col-0 plants).

238

239 **Ancestral and *Arabidopsis*-adapted mite strains induce similar JA-regulated**
240 ***Arabidopsis* defenses**

241 Suppression of induced plant defenses was proposed to be one of the mechanisms of
242 TSSM adaptation to new host plants (Blaazer *et al.*, 2018; Jonckheere *et al.*, 2018;

243 Jonckheere *et al.*, 2016; Villarroel *et al.*, 2016). One of the hallmarks of defense-
244 suppressing TSSM populations is the similarity of their performance on wild-type and
245 defense-deficient host plants (Kant *et al.*, 2008). However, *Arabidopsis*-adapted mites,
246 like the ancestral population, performed significantly better on *cyp79b2 cyp79b3* than
247 on Col-0 plants, Figure 2B and C, suggesting that mite adaptation to *Arabidopsis* is not
248 based on the suppression of host defenses. To corroborate the lack of defense
249 suppression in *Arabidopsis*-adapted mites we determined the expression levels of JA-
250 responsive *AOS, MYC2, CYP79B2,* and *CYP79B3* genes (labeled in red in Figure 1A) and
251 the abundance of JA, JA-Ile, and indol-3-ylmethylglucosinolate (I3M) (labeled in green in
252 Figure 1A) that were previously established as reliable markers of induced JA-regulated
253 *Arabidopsis* defenses against mite herbivory (Widemann *et al.*, 2021; Zhurov *et al.*,
254 2014). The expression levels and the abundance of defense-associated markers were
255 determined in Col-0 plants that were challenged with bean-a, *cyp*-a, or Col-a mites after
256 24 h of mite herbivory. As seen in Figure 3A, *cyp*-a and Col-a mites induced all JA-
257 regulated marker genes to similar or higher levels relative to non-adapted bean-a mites.
258 Likewise, JA, JA-Ile, and I3M accumulated at comparable levels in bean-a, *cyp*-a, and Col-
259 a challenged Col-0 plants (Figure 3B). Even though we can not rule out the possibility
260 that some plant pathways with minor effects on mite fitness may have been suppressed,
261 our data suggest that ancestral and *Arabidopsis*-adapted mites are exposed to similar
262 JA-regulated *Arabidopsis* defenses, shown to be both necessary and sufficient to protect
263 *Arabidopsis* plants against mite herbivory.
264

265 **Initial responses to *Arabidopsis* xenobiotics are similar in ancestral and *Arabidopsis*-**
266 **adapted mites**

267 The constitutive overexpression and/or increased transcriptional plasticity of genes
268 encoding xenobiotic-metabolizing enzymes have been previously implicated in the
269 evolution of TSSM resistance to pesticides (Kwon *et al.*, 2015; Van Leeuwen and
270 Dermauw, 2016; Van Leeuwen *et al.*, 2010) and mite adaptation to several plant hosts
271 (Agrawal *et al.*, 2002; Wybouw *et al.*, 2012; Wybouw *et al.*, 2014; Wybouw *et al.*, 2015).

272 We have previously identified forty genes, primarily encoding cytochrome P450
273 monooxygenases (P450s) and UDP-glycosyltransferase (UGTs), that were associated
274 with the initial TSSM responses to varying levels of Trp-derived defense compounds
275 (Zhurov *et al.*, 2014). To test if these genes may have contributed to mite adaptation to
276 *Arabidopsis*, we have arbitrarily chosen three genes within each class *(CYP392A1,*
277 *CYP392A16, CYP392D8, UGT201A2v2, UGT204B1*, and *UGT204A5)* and have used RT-
278 qPCR to determine their expression in bean-a, *cyp*-a, and Col-a mites that were kept on
279 bean plants for two generations before they were transferred to bean and *Arabidopsis*
280 plants with (Col-0) or without (*cyp79b2 cyp79b3*) Trp-derived defense compounds. On
281 the bean plants, all marker genes had similar and low expression levels in both bean-a
282 and *Arabidopsis*-adapted mites, Figure 4, indicating that none of the tested genes
283 underwent constitutive upregulation during mite adaptation to *Arabidopsis*. Consistent
284 with the previous report (Zhurov *et al.*, 2014), the transfer of bean-a mites to *cyp79b2*
285 *cyp79b3* or Col-0 plants resulted in induced expression of all tested genes. Their
286 expression was also induced in *cyp*-a and Col-a mites when they were shifted from bean
287 to *Arabidopsis* plants. Even though there was variability in the relative expression of
288 chosen *CYP* and *UGT* genes in these mites, their levels were either comparable or lower
289 than in bean-a mites (Figure 4). Thus, neither higher constitutive expression nor greater
290 transcriptional plasticity of tested *CYP* and *UGT* genes associates with the *Arabidopsis*-
291 adapted mites, demonstrating that: a) *Arabidopsis*-adapted mites retained
292 responsiveness to *Arabidopsis* xenobiotics, and b) the expression of tested mite genes
293 does not associate with TSSM adaptation to *Arabidopsis;* instead, it associates with
294 general xenobiotic responsiveness of bean-a, *cyp*-a, and Col-a mites to shift from bean
295 to *Arabidopsis* plants.
296
297 **Metabolic resistance underlies TSSM adaptation to *Arabidopsis***
298 To identify changes in the detoxification potential associated with mite adaptation to
299 *Arabidopsis*, we determined global enzymatic activity for three main protein families -
300 esterases, glutathione-S-transferases (GSTs), and P450s - in ancestral and *Arabidopsis*-

301 adapted mite populations feeding on bean and *Arabidopsis* plants. Overall, activities of
302 all three enzymatic classes were responsive to the host plant challenge: they were
303 lowest when mites fed on bean plants, and they progressively increased with the
304 complexity of *Arabidopsis* defenses, Supplemental Figure S2. However, of the three
305 classes of detoxification enzymes, P450 activity was consistently higher in Col-a mites
306 across all plant hosts, indicating that both constitutive and inducible levels of P450
307 activity increased in Col-a mites, (three-way ANOVA; plant host : mite strain interaction
308 $F = 103.93$, $p = 5.451e-16$, followed by Tukey's HSD test, $p < 0.01$, $n = 4$).

309 To test the requirement of esterase, GST, and P450 activities for TSSM
310 adaptation to *Arabidopsis*, we used S,S,S tributyl-phosphorotrithioate (DEF, an inhibitor
311 of esterase activity), diethyl maleate (DEM, an inhibitor of GST activity), and piperonyl
312 butoxide (PBO) and trichlorophenylpropynyl ether (TCPPE) (inhibitors of P450 activity)
313 to reduce the activity of the indicated enzymatic classes. If a particular class of enzymes
314 is required for mite adaptation to *Arabidopsis*, then the inhibition of its activity is
315 expected to restore the susceptibility of *Arabidopsis*-adapted mites to *Arabidopsis*
316 defense compounds. Concentrations of 2000 mg/L (DEM), 100 mg/L (DEF), 1000 mg/L
317 (PBO), and 1500 mg/L (TCPPE) were used as they cause <10% mite mortality
318 (Supplemental Figure S3), but were nevertheless capable of significantly reducing the
319 corresponding enzymatic activities in Col-a mites feeding on Col-0 plants (Figure 5A,C
320 and Supplemental Figure S4). As inhibitors do not affect all enzymes equally within the
321 targeted enzymatic class (Feyereisen, 2014), the lack of inhibitory effect on mite
322 performance is not strong evidence against the involvement of a particular enzymatic
323 class in mite host adaptation. Conversely, the significant decrease of mite performance
324 on a new host plant upon the application of a given inhibitor strongly supports the
325 requirement of enzyme(s) within the corresponding class for mite host adaptation.

326 The application of DEF reduced esterase activities by 32% but did not affect the
327 fitness of Col-a mites when feeding on either Col-0 or *cyp79b2 cyp79b3* plants
328 (Supplemental Figure S4), indicating that esterases may not be required for mite
329 adaptation to *Arabidopsis*. The application of DEM decreased the GST enzymatic activity

330 by 43% and had a minor but significant effect on the fecundity of Col-a mites
331 (Supplemental Figure S4), suggesting that GST activity is required for high Col-a
332 performance on *Arabidopsis*.

333 The application of PBO significantly reduced the P450 activity (25%) and
334 dramatically reduced (44%) the fecundity of Col-a mites feeding on Col-a host plants,
335 and to a lesser extent when they fed on *cyp79b2 cyp79b3* plants (Figure 5A,B). Since
336 PBO does not interfere with all P450s equally and also inhibits esterases in some
337 arthropods (Feyereisen, 2014), we applied another structurally unrelated P450 inhibitor,
338 TCPPE, to confirm that the observed effects were due to the decreased P450 activity.
339 TCPPE significantly inhibited P450 enzyme activity in Col-a mites (13%) and dramatically
340 reduced the fecundity of Col-a mites fed on Col-0 (39%) and *cyp79b2 cyp79b3* plants
341 (33%), (Figure 5C,D). In addition, the fitness of bean-a mites decreased by 13% when
342 they fed on *cyp79b2 cyp79b3* TCPPE-treated plants (Figure 5D).

343

344 **P450 activity is required for TSSM adaptation to *Arabidopsis***

345 As P450 inhibitors were applied directly to *Arabidopsis* leaves, there is a possibility that
346 PBO/TCPPE perturbed *Arabidopsis* defense physiology and only secondarily affected
347 mite fecundity. For example, the synthesis of JA metabolites (Aubert *et al.*, 2015; Heitz
348 *et al.*, 2012; Widemann *et al.*, 2015) and Trp-derived secondary metabolites (Hull *et al.*,
349 2000) occur via pathways that include many plant P450s. Therefore, to confirm the
350 requirement of mite P450s in TSSM adaptation to *Arabidopsis*, we used recently
351 developed RNAi gene silencing protocol in TSSM (Bensoussan *et al.*, 2020; Suzuki *et al.*,
352 2017) to silence the P450 pathway in mites.

353 Because the *CYP* (PF00067) gene family has over 100 *CYP* genes (Grbic *et al.*,
354 2011; Mistry *et al.*, 2020), it is difficult to characterize the involvement of individual
355 P450s in mite adaptation to *Arabidopsis* using RNAi. To circumvent this hurdle, we
356 instead silenced the expression of *NADPH-cytochrome P450 reductase* (*Tu-CPR*), a co-
357 enzyme required for the catalytic reactions carried out by microsomal P450s (Masters
358 and Okita, 1980). *Tu-CPR* (*tetur18g03390)* is a single copy gene (Grbic *et al.*, 2011)

359 constitutively expressed in all tissues of adult spider mites, including the midgut
360 epithelial and digestive cells (Figure 5F) where digestion and detoxification of dietary
361 xenobiotics are expected to take place (Bensoussan *et al.*, 2018). Application of dsRNA-
362 *Tu-CPR* to Col-a mites resulted in decreased expression of *Tu-CPR* (Figure 5G). Consistent
363 with the Tu-CPR function as an essential component of the P450 enzyme complex, P450
364 activity was reduced by 38% in the *Tu-CPR* silenced mites relative to mites exposed to
365 dsRNA-NC (Figure 5H). Strikingly, the silencing of *Tu-CPR* reduced the fecundity of Col-a
366 mites by almost 50% when they fed on Col-0 plants (Figure 5I). Similar phenotypes were
367 obtained upon the application of a second, independent, dsRNA-*Tu-CPR-1* fragment
368 (Supplemental Figure S5), confirming the specificity of the observed phenotypes to a
369 loss of *Tu-CPR* function and the requirement of P450 activity for the adaptation of Col-a
370 mites to *Arabidopsis*. The reduced P450 activity, through the application of P450
371 inhibitors or dsRNA-*Tu-CPR*, did not affect TSSM fecundity when mites fed on bean
372 plants (Figure 5E,I and Supplemental Figure S5), demonstrating that P450 activity is
373 specifically required for mites' ability to use *Arabidopsis* as a host.
374

375 **Discussion**

376 The innate ability to perceive and respond to plant defenses in a process of physiological
377 acclimation is assumed to enable broad-generalist herbivores to utilize a wide range of
378 host plants. On the other hand, genetic adaptation is presumed to enable specialist
379 herbivores to evolve novel adaptation traits that efficiently counteract the defenses of a
380 narrow range of host plants (Barrett and Heil, 2012; Despres *et al.*, 2007; Heidel-Fischer
381 and Vogel, 2015). However, our understanding of the host-shift responses in composite
382 generalist herbivores that exist as complexes of host-specialized populations is not well
383 understood. *Tetranychus urticae* is an example of a composite generalist herbivore that
384 feeds on over thousand plant hosts. Individual mite populations thrive on a subset of
385 potential hosts but can rapidly adapt to new plant hosts (Agrawal *et al.*, 2002; Fellous *et*
386 *al.*, 2014; Fry, 1989; Gould, 1979; Magalhaes *et al.*, 2007; Wybouw *et al.*, 2015). Thus,
387 mite host adaptability is one of the key features underlying *T. urticae* extremely wide
388 host range.

389 *T. urticae*, like other polyphagous herbivores, acclimates to host shift by
390 reprogramming its salivary and detoxification complements (Figure 4 and (Dermauw *et*
391 *al.*, 2013; Jonckheere *et al.*, 2018; Jonckheere *et al.*, 2016; Villarroel *et al.*, 2016;
392 Wybouw *et al.*, 2015; Zhurov *et al.*, 2014)). However, exposure to initially unfavorable
393 host plants over several generations enables *T. urticae* to dramatically increase its
394 performance (Figure 2 and (Fry, 1989; Gould, 1979; Magalhaes *et al.*, 2007; Wybouw *et*
395 *al.*, 2015). Here, we used *Arabidopsis* and bean-adapted (bean-a) mites to investigate
396 mechanisms that mediate the formation of host-adapted populations in this generalist
397 herbivore. *Arabidopsis* is a non-preferred host for the bean-a mite population, Figure 1.
398 However, under experimental selection, bean-a mites gave rise to *Arabidopsis*-adapted
399 *cyp*-a and Col-a populations, Figure 2. Matching the complexity of *Arabidopsis* defenses,
400 *Arabidopsis*-adapted populations evolved adaptation traits capable of counteracting
401 *CYP79B2 CYP79B3*-independent *Arabidopsis* defenses (present in both *cyp*-a and Col-a
402 mites) and Trp-derived defenses (present only in Col-a mites). Remarkably, these
403 complex adaptation traits were established within 25 generations of mite *Arabidopsis*
404 selection.
405 Suppression of plant defenses has been proposed to be one of the mechanisms
406 of mite host adaptation (Blaazer *et al.*, 2018; Jonckheere *et al.*, 2018; Jonckheere *et al.*,
407 2016; Villarroel *et al.*, 2016). Attenuation of host induced responses has been described
408 for several spider mites species in their interactions with the *Solanum lycopersicum*
409 (tomato) plant host (Glas *et al.*, 2014; Kant *et al.*, 2008; P. Godinho *et al.*, 2016;
410 Sarmento *et al.*, 2011). The prevalence of the ability to suppress plant responses across
411 tomato-adapted *T. urticae* populations is currently not known, nor is its presence in
412 other *T. urticae* host-adapted populations. Even though the interference with either JA-
413 biosynthesis or its signalling could have been an effective way to attenuate a whole
414 range of *Arabidopsis* defense compounds, (Figure 1, (Widemann *et al.*, 2021; Zhurov *et*
415 *al.*, 2014)), our data suggest that *Arabidopsis*-adapted mites did not interfere with JA-
416 regulated *Arabidopsis* defenses. *Arabidopsis*-adapted mites, just like the ancestral bean-
417 a population, had greater fitness on *cyp79b2 cyp79b3* than on Col-0 plants (Figure 2).

418 They also induced the expression of JA-responsive genes and accumulated I3M to levels
419 similar to those induced by the bean-a mites (Figure 3). This is surprising, as ancestral
420 bean-a strain used in this study previously gave rise to tomato-adapted populations that,
421 in the process, gained the ability to suppress tomato mite-induced responses (Wybouw
422 *et al.*, 2015), raising the question if and how host defenses affect the mechanism of
423 TSSM adaptation. Instead, we provided multiple independent lines of evidence
424 indicating that *Arabidopsis*-adapted mites evolved metabolic resistances to counteract
425 *Arabidopsis* defenses.

426 The pharmacological experimental treatments identified the requirement of
427 GSTs for the fitness advantage of Col-a over bean-a mites when they fed on Col-0 plants
428 (Supplemental Figure S4). GST-based detoxification has been associated with the ability
429 of numerous generalist herbivores to counteract the anti-herbivore effects of
430 glucosinolates, considered to be the major class of *Arabidopsis* defense compounds
431 (Gloss *et al.*, 2014; Jeschke *et al.*, 2016; Schramm *et al.*, 2012; Wadleigh and Yu, 1988).
432 Aliphatic and indole glucosinolates are the most abundant classes of these compounds
433 in *Arabidopsis* (Brown *et al.*, 2003). Herbivores have differential susceptibility to
434 aliphatic and indole glucosinolates, so that some are exclusively affected by the aliphatic
435 (Müller *et al.*, 2010), some by the indole (Kim and Jander, 2007), and some by both
436 classes of glucosinolates (Jeschke *et al.*, 2017; Müller *et al.*, 2010). Aliphatic
437 glucosinolates are ineffective in restricting mite herbivory (Zhurov *et al.*, 2014). Instead,
438 the antifeedant effects of indole glucosinolates on *T. urticae* have been recently
439 characterized (Widemann *et al.*, 2021), raising a possibility that *Arabidopsis* defensive
440 indole glucosinolate breakdown products may be detoxified by mite adaptation-
441 associated GST(s).

442 The pharmacological and RNAi reverse genetics experiments pointed to the
443 critical requirement of P450 activities for the adaptation of Col-a mites to *Arabidopsis*
444 (Figure 5, Supplemental Figure 4). We identified two distinct modes of P450 activities.
445 One corresponds to the initial mite response to the shift from bean to *Arabidopsis* that
446 is similar in both ancestral and *Arabidopsis*-adapted mites (Figure 4). The inhibition of

447 these P450s had a limited but significant effect on the fecundity of bean-a ancestral
448 TSSM population when these mites were challenged on the *Arabidopsis* host (Figure 5D).
449 As bean-a mites had no prior exposure to *Arabidopsis* plants, the effect of P450
450 inhibitors on their fecundity uncovers the limited contribution of initially induced P450s
451 to mite fitness on *Arabidopsis.* Since *Arabidopsis*-adapted mites retained responsiveness
452 to shift from bean to *Arabidopsis* plants, Figure 4, it is expected that these P450s enable
453 limited mite adaptation to *Arabidopsis* defenses ((Zhurov et al., 2014) and Figure 5). This
454 is consistent with the physiological acclimation described for broad-generalists whereby
455 exposure to plant xenobiotics is assumed to result in the transcriptional induction of
456 genes encoding enzymes that recognize a wide range of substrates and have low
457 enzymatic catalytic activity (Halon *et al.*, 2015; Li *et al.*, 2004; Mathers *et al.*, 2017;
458 Sasabe *et al.*, 2004; Shi *et al.*, 2018; Snoeck *et al.*, 2019; Wang *et al.*, 2018; Wen *et al.*,
459 2006). The other more significant contribution of P450-mediated metabolic resistance is
460 realized through the action of highly effective P450 detoxification responses against
461 *Arabidopsis* defenses that are exclusively associated with mite adaptation to *Arabidopsis.*
462 The reduction of P450 activity in Col-a mites diminished their fitness advantage over
463 ancestral bean-a mites, demonstrating that adaptation-associated P450 activity is the
464 main contributor to mite resistance to *Arabidopsis* defenses (Figure 5B,D,I and
465 Supplemental Figure S5). The inhibition of P450 activity reduced Col-a mite fecundity
466 when they fed on both Col-0 and *cyp79b2 cyp79b3* plants (Figures 5B,D), suggesting that
467 adaptation-associated P450s mainly counteract CYP79B2 CYP79B3-independent
468 *Arabidopsis* defenses whose identity is currently not known. However, the requirement
469 of P450 activity for the adaptation of green peach aphid *M. persicae* to *Arabidopsis*
470 indole glucosinolates (Ji *et al.*, 2021) raises the possibility that mite adaptation-
471 associated P450s may also counteract the toxicity of indole glucosinolate-associated
472 defensive compounds.

473 In summary, as demonstrated here for the *Arabidopsis*-adapted mite
474 populations, *T. urticae* can evolve resistance against complex plant defenses within a
475 mere 25 generations. *Arabidopsis*-adapted mites retained the ability to acclimate to

476 shift from bean to *Arabidopsis*. Such transcriptional plasticity, characteristic of broad-
477 generalists, is shared between ancestral and *Arabidopsis*-adapted mites and has a small
478 contribution to mite fitness on *Arabidopsis*. At present, it is not clear if these initial
479 responses are common stress/xenobiotic responses to any novel host, nor if they are
480 required for mite host adaptation. If they are, they may provide initial fitness benefit
481 upon mites' shift to a new host that may facilitate the evolution of the host adaptation
482 traits. We further identified the highly effective P450 detoxification responses that are
483 required for mite adaptation to *Arabidopsis*. This pattern of metabolic counteraction is
484 reminiscent of strategies deployed by host-specialised herbivores, in which host
485 adaptation is associated with either nucleotide substitutions that enhance the enzyme
486 activity (Li *et al.*, 2007; Schuler, 2011) or with the overexpression of detoxification
487 genes (Bass *et al.*, 2013). The *CYP* gene family underwent a lineage-specific expansion in
488 TSSM and consists of 115 genes in bean-a mites (Grbic *et al.*, 2011; Mistry *et al.*, 2020).
489 Intraspecific genetic variability is expected at *CYP* loci as some appear as clusters of
490 duplicated genes that are prone to the formation of chimeric genes or copy number
491 variation. Comparative genome and transcriptome analysis of *Arabidopsis*-adapted and
492 ancestral TSSM populations may identify candidate adaptation-required *CYP* gene(s). In
493 humans, for example, only 15 out of 57 P450s are involved in xenobiotic metabolism
494 (Handschin and Meyer, 2003). The comparative sequence and functional analysis of
495 candidate *CYPs* will reveal if adaptation-required P450 activity is achieved by altering
496 gene regulation, copy number, or through substitution(s) that increase enzyme activity
497 and if these changes resulted from the selection within the genetic pool of founding
498 population, through *de novo* mutations or epigenetic changes.
499

500 **Materials and Methods**
501

502 **Plant growing and mite rearing conditions**

503 Columbia-0 (Col-0) seeds were obtained from the Arabidopsis Biological Resource
504 Center (ABRC, Ohio State University), *cyp79b2 cyp79b3* (Zhao *et al.*, 2002) from B. A.
505 Halkier (University of Copenhagen, Denmark), *myc2 myc3 myc4* (Fernandez-Calvo *et al.*,

506 2011), from R. Solano (Universidad Autónoma de Madrid) and bean (*Phaseolus vulgaris*,
507 cultivar California Red Kidney) from Stokes, Thorold, Ontario, Canada. Plants were
508 grown under 100 to 150 µmol m^{-2} sec^{-1} cool-white fluorescent light at 24°C with a 10-
509 h/14-h (light/dark) photoperiod in controlled growth chambers. *Tetranychus urticae*,
510 London reference strain (Grbic *et al.*, 2011), were reared on bean plants at 24°C, 60%
511 relative humidity, and with a 16-h/8-h (light/dark) photoperiod for >10 years.
512
513 **Experimental evolution**
514 Approximately 1,000 randomly chosen adult bean-a female mites were transferred to 3-
515 week-old *cyp79b2 cyp79b3* or Col-0 *Arabidopsis* plants that were replaced biweekly.
516 Mite populations were allowed to propagate for 18 months, generating six selected
517 lines: #1-#3 independent lines each selected on *cyp79b2 cyp79b3* and Col-0 plants,
518 referred to as *cyp*-a and Col-a lines respectively. Selected lines were reared on their
519 corresponding plant hosts under the same conditions as the ancestral strain. *cyp*-a (#3)
520 and Col-a (#3) were used for the follow-up experiments.
521
522 **Mite performance analysis**
523 Twenty 3-day-old adult female mites were transferred to each of the experimental
524 plants (bean, *cyp79b2 cyp79b3* and Col-0) and total population size at seven days post-
525 inoculation was evaluated in direct (mites were moved directly from their rearing hosts
526 to experimental plants) and indirect (mites were reared on bean plants for two
527 generations before their transfer to the experimental plants) transfer regimes. The
528 experiment was performed in four biological replicates for each selected line.
529 Differences between selected line population sizes were determined using a one-way
530 ANOVA using mite strain as the main effect, followed by Tukey's honestly significant
531 difference (HSD) test. All other experiments were performed with mites that were
532 propagated for two generations on bean plants, thus, in indirect transfer regime.
533
534 **Plant damage analysis**

535 Leaf damage of *cyp79b2 cyp79b3* and Col-0 *Arabidopsis* plants upon feeding of bean-a,
536 *cyp*-a, and Col-a mite strains was performed as previously described (Zhurov *et al.*,
537 2014). Briefly, ten adult female mites were placed on the rosette of *Arabidopsis* plants
538 and allowed to feed for 3 days before plants were cut at the base of the rosette. The
539 adaxial side of the rosette was scanned using a Canon CanoScan 8600F model scanner
540 (Canon U.S.A. Inc., Melville, NY, U.S.A) at a resolution of 1200 dpi and a brightness
541 setting of +25. Scanned plants were saved as .jpg files for subsequent analysis in Adobe
542 Photoshop 5 (Adobe Systems, San Jose, CA). The experiment was performed using six
543 biological replicates/trial, and in three experimental trials, n = 18. Differences in plant
544 damage between mite strains on different hosts were determined using a three-way
545 ANOVA, using mite strain, plant host, and experimental trial as main effects, including
546 interaction terms, followed by Tukey's HSD test. The inclusion of experimental trial and
547 its potential interactions with main effects was used both to control for the effect of
548 trial and to test for reproducibility across trials, as suggested by (Brady *et al.*, 2015). This
549 statistical approach was used in all following analyses.
550
551 **Mite fecundity assay**
552 Fecundity over two days of bean-a mites on Col-0, *cyp79b2 cyp79b3,* and *myc2 myc3*
553 *myc4*, and fecundity over six days of bean-a, *cyp*-a, and Col-a mites on *cyp79b2 cyp79b3*
554 or Col-0 *Arabidopsis* plants were assessed as previously described (Widemann *et al.*,
555 2021). The experiment included five to ten biological replicates for each treatment that
556 were repeated in three experimental trials. A two- and three-way ANOVA was
557 performed respectively, using the mite strain, plant genotype, and experimental trial as
558 main effects including relevant two-way interaction terms when required. For the mite
559 fecundity assay on MeJA-treated Col-0 leaves, the leaves from five-week-old Col-0
560 plants were sprayed 6 times over 48 h with methyl jasmonate (MeJA) (Sigma-Aldrich,
561 Cat # 392707, 500 µM in ethanol 0.4 % (v/v)), as previously described (Feldberg *et al.*,
562 2009). On the second day of treatment, fully-elongated leaves were detached and a day
563 later infested with 10 adult female bean-a mites whose fecundity was recorded after 24

564 h. The experiment included nine biological replicates and was repeated in two
565 experimental trials. Differences in fecundity were detected by a two-way ANOVA, using
566 the treatment and experimental trial as main effects including an interaction term.
567
568 **Real-Time Quantitative Reverse Transcription-PCR and metabolic analyses**
569 For plant marker gene and metabolite analyses, ten three-day-old adult female bean-a,
570 *cyp*-a, and Col-a mites, propagated for two generations on bean plants, were allowed to
571 feed on Col-0 plants for 24 h after which whole rosettes were collected. For TSSM
572 marker gene analysis, samples of 100 three-day-old adult female bean-a, *cyp*-a, and Col-
573 a mites, initially propagated for two generations on bean plants and then transferred to
574 bean, *cyp79b2 cyp79b3,* or Col-0 plants for 24 h, were collected. Both experiments were
575 replicated in three biological replicates/trial and three independent trials. Preparation of
576 RNA, cDNA, and the real-time quantitative reverse transcription-PCR analysis was
577 performed as previously described (Zhurov *et al.*, 2014). Primer sequences and
578 amplification efficiencies (E) used in qPCR are shown in Supplemental Table 1. *PEROXIN4*
579 (*AT5G25760*) and *RP49* (*tetur18g03590*) were used as the reference genes for
580 *Arabidopsis* and mite genes, respectively. Ct values of three technical replicates were
581 averaged to generate a biological replicate Ct value. Plotting and statistical analysis were
582 performed as described previously (Rieu and Powers, 2009). The quantification of plant
583 metabolites (JA, JA-Ile, and I3M) was performed as described in Zhurov et al., 2014.
584 Briefly, jasmonates (JA and JA-Ile) were analyzed in aqueous extracts by mass
585 spectrometry monitoring their specific precursor-to-product ion transition in a triple
586 quadrupole mass spectrometer (Micromass Ltd. UK). Quantitation was achieved after
587 external calibration with standards of known amount considering the analyte/internal
588 standard ratio (dihydrojasmonic acid, added before extraction). Moreover, 3-
589 indolylmethyl glucosinolate (I3M) was identified and analyzed following a non-targeted
590 approach using a hybrid quadrupole-time-of-flight (QqTOF) mass spectrometer.
591 Identification of I3M was performed after inspection of its specific mass spectrum in
592 negative electrospray ionization mode: 447.053 $[M-H]^-$, 253.03 $[C_{10}H_9N_2O_4S]^-$, 96.95

593 $[HSO_4]^-$. Relative quantitation of I3M was attained by dividing peak areas of I3M and
594 internal standard (biochanin A, 283.07 $[M-H]^-$). The resulting value was again divided by
595 the actual sample weight expressed in mg. Mite marker gene data were analyzed by a
596 two-way ANOVA with mite strain and plant host as main effects including an interaction
597 term, followed by Tukey's HSD test. For plant marker gene and metabolite analysis, the
598 data were analyzed by a two-way ANOVA, using mite strain and experimental trial as
599 main effects including an interaction term, followed by Tukey's HSD test. For a graphical
600 representation of the expression of plant marker genes, the NRQ data were further
601 normalized to the 'no mite' control sample, which was therefore set to one. Thus, these
602 results represent fold change differences in the expression of marker genes relative to
603 'no mite' control.
604
605 **Determination of cytochrome P450 monooxygenase (P450), esterase and glutathione-**
606 **S-transferase (GST) activity**
607 To perform enzymatic activity assays, 3-day-old spider mite females of each Col-a, *cyp*-a,
608 and bean-a strains, reared for two generations on bean plants, were placed on bean,
609 *cyp79b2 cyp79b3,* or Col-0 plants. After 24 h, 200 females from each treatment were
610 collected and were used for protein extraction and enzymatic assays. Mites were
611 homogenized in 700 µL of 100 mM phosphate buffer, pH 7.6. The concentration of total
612 protein mite extracts was determined using the Quick Start Bradford Protein Assay
613 (Quick start Bradford 1x dye reagent, Bio-Rad, Cat# 500-0205), with Bovine Serum
614 Albumin (Sigma-Aldrich, Cat # A7906) as the standard. Twenty, 10, and 10 µg of protein
615 per reaction were used for measuring the enzymatic activity of glutathione-S-
616 transferases (GSTs), P450 monooxygenases, and esterases, respectively. The enzymatic
617 activities were assessed spectrophotometrically using 1-chloro-2,4-dinitrobenzene
618 (CDNB) (for GSTs) (Habig and Jakoby, 1981), 7-ethoxy-4-trifluoromethylcoumarin (7-EFC)
619 (for P450s) (Buters *et al.*, 1993), and 4-nitrophenyl acetate (pNPA) (for esterases) (Park
620 *et al.*, 1961), as substrates. For GST and esterases, the linearity of the reaction was
621 determined by plotting the absorbance values against time over 5 minutes period and

622 calculation of samples activity was performed in the linear range. All assays were
623 corrected for the occurrence of non-enzymatic substrate transformation in blank
624 samples in which the protein solution was replaced by the buffer. Enzymatic activities
625 were determined in four independent samples with three technical replicates per
626 sample. Differences in enzymatic activity between mite strains on different plant hosts
627 were detected using a two-way ANOVA, with mite strain and plant host as main effects
628 including an interaction term, followed by Tukey's HSD test.
629
630 **Application of enzyme inhibitors**
631 Solutions of 2000 mg/L diethyl maleate (DEM, an inhibitor of glutathione S-transferase
632 (GST) activity), 100 mg/L S,S,S tributyl-phosphorotrithioate (DEF, an inhibitor of
633 esterase activity), 1000 mg/L piperonyl butoxide (PBO) and 1500 mg/L
634 trichlorophenylpropynyl ether (TCPPE) (inhibitors of P450 activities) were used as they
635 cause <10% mortality of bean-a mites (Supplemental Figure S3). Detached leaves of Col-
636 0 *Arabidopsis* plants were dipped in one of the DEF, DEM, PBO or TCPPE solutions or
637 water and acetone 0.1 % (v/v) as control. Upon drying, leaves were infested with either
638 one adult bean-a or Col-a female mite and the number of eggs laid by each female per
639 day was recorded for six days, or with ten 2-day-old adult Col-a female mites that were
640 collected after 24 h. A pool of 100 spider mites was used to measure GST, esterase, and
641 P450 activities in DEM-, DEF-, PBO- and TCPPE-treated spider mites, respectively.
642 Fecundity was determined in three independent experimental trials with ten replicates
643 per trial. Enzymatic activities were determined in five independent samples with three
644 technical replicates per sample. Differences between means of fecundity and enzymatic
645 activities of control and inhibitor-treated samples were calculated using unpaired
646 Student's t-tests.
647
648 ***In situ* hybridization**
649 DIG-labelled probes were produced and the whole-mount *in situ* hybridization was
650 performed according to previously published methods (Dearden *et al.*, 2002). Images

651 were collected using a Zeiss AxioCam HRc 412-312 camera mounted on a Zeiss Axioplan
652 II microscope.
653
654 **RNAi of *Tu-CPR***
655 Two non-overlapping fragments (*Tu-CPR*, 645 nt) and (*Tu-CPR-F1*, 564 nt),
656 complementary to the coding region of *Tu-CPR (tetur18g03390*) and dsRNA
657 complementary to a 382 bp non-transcribed intergenic fragment (1690614-1690995 of
658 the genomic scaffold 12) used as a negative control dsRNA (referred to as NC) were
659 synthesized using primers listed in Supplementary Table 1 as described in (Suzuki *et al.*,
660 2017). A BLAST search against the *T. urticae* genome confirmed that dsRNA sequences
661 are unique. dsRNA solutions at concentrations of 500 ng/µL were supplemented with 6%
662 (v/v) blue dye erioglaucine (McCormick, Sparks Glencoe, MD). Newly molted adult
663 female mites were soaked in dsRNA/dye solutions at 20°C for 24 h (Suzuki *et al.*, 2017).
664 Post soaking, mites with visible blue dye in the posterior midgut were selected and were
665 transferred in batches of 10 to either detached Col-0 leaves or bean leaf disks.
666 Fecundity was determined in three independent experimental trials with ten
667 replicates/trial as a number of eggs deposited by individual female mite on the 3rd and
668 4th day post soaking. For the analysis of the *CPR* expression, 100 adult female mites
669 were treated with either dsRNA-*Tu-CPR,* dsRNA-*Tu-CPR-1*, or dsRNA-NC. Mites were
670 allowed to feed on Col-0 leaves for four days. Thereafter, mites were selected based on
671 visual phenotype (spotless) and were used for the analysis of *CPR* expression levels. The
672 RT-qPCR was performed in eight (for dsRNA-*CPR*) and three (for dsRNA-*CPR-1*)
673 experimental replicates, as described above. For the analysis of P450 activity in *CPR*
674 silenced mites, 100 adult female mites were treated with either dsRNA-*Tu-CPR,* dsRNA-
675 *Tu-CPR-1*, or dsRNA-NC. After four days of feeding on Col-0 leaves, mites were collected
676 and the P450 activity was determined in five independent samples with three technical
677 replicates per sample, as described above. Differences in fecundity between dsRNA-
678 treated mites were detected using a two-way ANOVA, with mite treatment and
679 experimental trial as main effects including an interaction term, followed by Tukey's

680 HSD test. Enzyme activity and RT-qPCR expression levels were compared between
681 dsRNA-treated mites using unpaired Student's t-tests. Independent mite samples were
682 used for the fecundity assay and the analysis of transcript levels and P450 activity.
683
684

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689

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691

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998

999 **Figure Legends**

1000 **Figure 1. Two-spotted spider mite (TSSM) *Tetranychus urticae* induces complex**
1001 **defense responses in *Arabidopsis*. A**, A simplified schematic of TSSM feeding-induced
1002 *Arabidopsis* defense responses. **B**, The fecundity of bean-reared TSSM (ancestral
1003 population) upon feeding on MeJA treated Col-0 plants (left) and on Col-0 wild type,
1004 *cyp79b2 cyp79b3* and *myc2 myc3 myc4* mutant plants *(myc2,3,4)* (right). Data are
1005 presented as mean number of eggs laid by a female mite per day ± SEM. Left, n = 18;
1006 asterisk indicates a significant difference between treated and control samples (ANOVA:
1007 ***P < 0.001). Right, n = 15 Different letters represent significant difference between
1008 means (Tukey's HSD test, α = 0.05).

1009

1010 **Figure 2. *Tetranychus urticae* can adapt to complex array of *Arabidopsis* induced**
1011 **defenses**. **A**, A schematic of the experimental evolution procedure. **B**, Performance of
1012 ancestral and selected populations on bean (top), *cyp79b2 cyp79b3* (middle) and Col-0
1013 (bottom) plants. Data are from the indirect transfer experiment where *Arabidopsis*
1014 selected TSSM were reared for two generations on bean before transfer to the
1015 experimental plants. Data represent the mean size of population (the sum of eggs,
1016 larvae, nymphs, and adults) derived from twenty adult female mites at seven days post
1017 infestation ± SEM, n = 4. Different letters represent significant difference between
1018 means (Tukey's HSD test, α = 0.05). Data from direct transfer experiments where
1019 *Arabidopsis* selected TSSM were directly transferred from their rearing to the
1020 experimental plants is shown in Supplemental Figure S1. **C**, Fecundity of the ancestral
1021 (bean-a) and populations #3 of *Arabidopsis*-selected mites (*cyp*-a and Col-a) upon
1022 feeding on Col-0 and *cyp79b2 cyp79b3* plants. Data are presented as mean number of
1023 eggs laid by a female mite per day ± SEM, n = 30. **D**, Leaf damage of Col-0 and *cyp79b2*
1024 *cyp79b3 Arabidopsis* plants upon herbivory of ancestral (bean-a), and populations #3 of
1025 *Arabidopsis*-selected mites (*cyp*-a and Col-a). Data represent mean area of chlorotic
1026 spots (mm^2) ± SEM, n = 18. Different letters represent significant differences between
1027 means (Tukey's HSD test, α = 0.05). Individual sample values are shown as open circles
1028 for $n \leq 10$.

1029

1030 **Figure 3. Bean-a, *cyp*-a and Col-a mites induce similar *Arabidopsis* defense responses.**
1031 **A**, Expression of *AOS, CYP79B2, CYP79B3* and *MYC2* genes in Col-0 leaves in response to
1032 bean-a, *cyp*-a and Col-a mite feeding. Shown are means ± SEM of fold changes of
1033 expression levels detected by RT-qPCR relative to no mite control, *n* = 9. Primer
1034 sequences and amplification efficiencies (E) used in qPCR are shown in Supplemental
1035 Table 1. *PEROXIN4* (*AT5G25760*), was used as the reference gene. **B**, Levels of JA and JA-
1036 Ile (ng g^{-1} fresh weight), and relative level of the indol-3-ylmethyl glucosinolate (I3M,
1037 shown as normalized peak area), in 3-week-old Col-0 plants after herbivory of bean-a,
1038 *cyp*-a and Col-a mites for 24 h. Values are means ± SEM, *n* = 6. Different letters
1039 represent significant differences between means (Tukey's HSD test, α = 0.05). Individual
1040 sample values are shown as open circles for $n \leq 10$.

1041

1042 **Figure 4. Levels of *Arabidopsis*-induced mite *cytochrome P450* (*CYP*) and *UDP-***
1043 ***glycosyltransferase* (*UGT*) genes. A,** *CYP392A1* (*tetur07g06410*), *CYP392A16*
1044 (*tetur06g04520*) and *CYP392D8* (*tetur03g05070*). **B**, *UGT201A2v2* (*tetur02g02480*),
1045 *UGT204B1* (*tetur02g09830*) and *UGT204A5* (*tetur05g00090*). Shown are means ± SEM
1046 for relative quantity of expression normalized to *RP49* (*tetur18g03590*) reference gene,
1047 *n* =3. Primer sequences and amplification efficiencies (E) used in qPCR are shown in
1048 Supplemental Table 1. Different letters represent significant differences between means
1049 (Tukey's HSD test, α = 0.05). Individual sample values are shown as open circles for $n \leq$
1050 10.

1051

1052 **Figure 5. Cytochrome P450 activity is required for *Tetranychus urticae* adaptation to**
1053 ***Arabidopsis*. A,C** The activities of cytochromes P450 in Col-a mites feeding on Col-0
1054 plants after the application of piperonyl butoxide (PBO, in **A**) and
1055 trichlorophenylpropynyl ether (TCPPE, in **C**) inhibitors of P450 activity. Data are
1056 represented as mean ± SEM, *n* = 5. **B**,**D** The effects of PBO (**B**) and TCPPE (**D**) on
1057 fecundity of bean-a and Col-a mites upon feeding on Col-0 and *cyp79b2 cyp79b3* plants.

1058 **E**, The effect of TCPPE treatment on fecundity of bean-a and Col-a mites upon feeding
1059 on bean plants. Fecundity in **B**,**D** and **E** was measured over six days and data are
1060 presented as mean number of eggs laid by a female mite per day ± SEM; *n* = 30. **F**,
1061 Whole-mount *in situ* localization of *Tu-CPR* in *T. urticae* (i, ii, iv-vii, anti-sense probe). (i)
1062 adult female, (ii) adult male, (iii) adult female (hybridization with sense probe,
1063 background control). (iv-vii) enlarged views of (iv) epithelium, (v) ovaries and (vi and vii)
1064 digestive cells of adult female, taken from independently stained mites. **G**-**I**, Effects of
1065 dsRNA-*Tu-CPR*. **G**, Relative level of *Tu-CPR* transcript normalized with *RP49* in dsRNA-
1066 treated Col-a mites (mean ± SEM, *n* = 8). **H**, The activity of cytochromes P450 in dsRNA-
1067 treated Col-a mites upon feeding on Col-0 plants (mean ± SEM, *n* = 5). **I**, Fecundity of
1068 dsRNA-treated Col-a mites feeding on Col-0 or bean plants. Fecundity was measured
1069 over two days (3rd and 4th dpi) and data are presented as mean number of eggs laid by a
1070 female mite per day ± SEM, *n* = 30 for Col-0 plants and *n* = 12 for bean plants. See also
1071 Supplemental Figure S5. Asterisks indicate a significant difference between treated and
1072 control samples (unpaired Student's t test: *P < 0.05, **P < 0.01, ***P < 0.001). Scale
1073 bars in **F**: (i-iii) 20 µm; (iv-vii) 100 µm. Individual sample values are shown as open circles
1074 for $n \leq 10$.

1075 **Supplemental Data**

1076 **Supplemental Table 1.** Gene-specific primer sequences used for real-time quantitative
1077 RT-PCR.

1078

1079 **Supplemental Figure S1**. Experimental evolution of *Tetranychus urticae* adaptation to
1080 *Arabidopsis*. Performance of ancestral and selected populations on bean (top), *cyp79b2*
1081 *cyp79b3* (middle) and Col-0 (bottom) plants. Mites were transferred directly from their
1082 respective rearing hosts to experimental bean, *cyp79b2 cyp79b3* and Col-0 plants. The
1083 performance was measured as the size of total population derived from twenty adult
1084 female mites and seven days post infestation. Data are represented as mean ± SEM, *n* =
1085 4. Statistics were performed on total population counts. Different letters represent
1086 significant difference between means (Tukey's HSD test, α = 0.05). Individual sample
1087 values are shown as open circles for n ≤ 10.

1088

1089 **Supplemental Figure S2**. The activities of cytochromes P450 (**A**), glutathione-S-
1090 transferases (**B**) and esterase (**C**) in bean-a, *cyp*-a and Col-a mites (reared on beans for
1091 two generations) feeding on bean, *cyp79b2 cyp79b3* or Col-0 plants. Data are
1092 represented as mean ± SEM, *n* = 4. Different letters represent significant differences
1093 between means (Tukey's HSD test, α = 0.05), following three-way ANOVA. Plant host -
1094 mite strain interaction was significant for the activity of cytochromes P450 (F = 103.93, P
1095 = 5.451e-16). Individual sample values are shown as open circles for n ≤ 10.

1096

1097 **Supplemental Figure S3.** Selection of experimental concentration of enzyme inhibitors.
1098 Data are presented as mean mortality of a female mite at 3 dpi ± SEM (for treatment
1099 and control *n* = 10 for PBO, DEM and DEF experiments, and *n* = 9 for TCPPE experiment).
1100 Individual sample values are shown as open circles for n ≤ 10.

1101

1102 **Supplemental Figure S4**. The requirements of esterase and glutathione-S-transferase
1103 (GST) activities for *Tetranychus urticae* adaptation to *Arabidopsis*. The GST (**A**) and

1104 esterase (**C**) activities in Col-a mites feeding on Col-0 plants after the application of
1105 diethyl maleate (DEM, an inhibitor of GST activity, in **A**) and S,S,S tributyl-
1106 phosphorotrithioate (DEF, an inhibitor of esterase activity, in **C**). Data are represented as
1107 mean ± SEM, *n* = 5. **B** and **D**, Effects of DEM (in **B**) and DEF (in **D**) treatments on
1108 fecundity of bean-a and Col-a mites upon feeding on Col-0 and *cyp79b2 cyp79b3* plants.
1109 Fecundity was assessed as mean number of eggs laid by a female mite in six days ± SEM,
1110 *n* = 30. Asterisks in panels **A**-**C** indicate a significant difference between treated and
1111 control samples (unpaired Student's t test: *P < 0.05, **P < 0.01). Individual sample
1112 values are shown as open circles for n ≤ 10.
1113
1114 **Supplemental Figure S5.** RNAi silencing of *Tu-CPR* gene. **A**, A schematic of the *Tu-CPR*
1115 locus. DNA sequences used for the generation of dsRNA-*Tu-CPR* are shown in red
1116 (fragment CPR, 645 bp), and green (fragment CPR-1, 564 bp). UTR and coding sequences
1117 are shown as light and dark blue boxes, respectively. **B**, The effect of dsRNA-*Tu-CPR*.
1118 Relative expression of *Tu-CPR* normalized with *RP49* in dsRNA treated Col-a mites (mean
1119 ± SEM, *n* = 8, Student's t test, ns). **C**, Fecundity of dsRNA treated Col-a mites feeding on
1120 Col-0 and bean plants. Fecundity was measured over two days (3 and 4 dpi) and data are
1121 presented as mean number of eggs laid by a female mite per day ± SEM, *n* = 30 for Col-0
1122 plants and *n* = 12 for bean plants. Data are represented as mean ± SEM (Student's t test
1123 *** P < 0.001). Individual sample values are shown as open circles for n ≤ 10.
1124

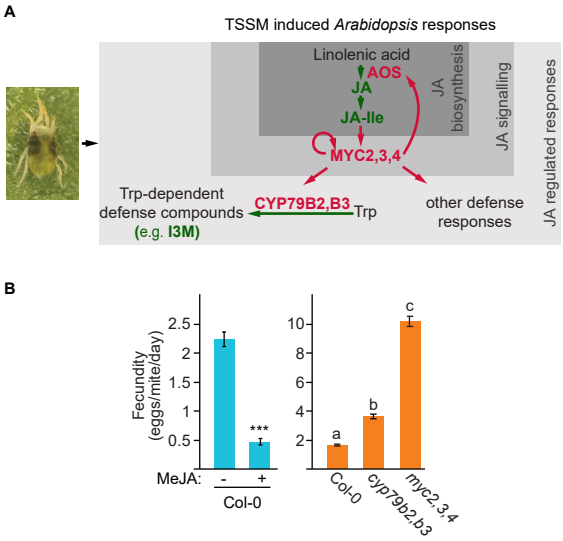

A
TSSM induced *Arabidopsis* responses
Linolenic acid
AOS
JA
JA-Ile
JA biosynthesis
JA signaling
JA regulated responses
MYC2,3,4
Trp-dependent defense compounds (e.g. I3M)
CYP79B2,B3
Trp
other defense responses
B
Fecundity (eggs/mite/day)
2.5
2
1.5
1
0.5
***
MeJA:
-
+
Col-0
10
8
6
4
2
a
b
c
Col-0
cyp79b2,b3
myc2,3,4

Figure 1

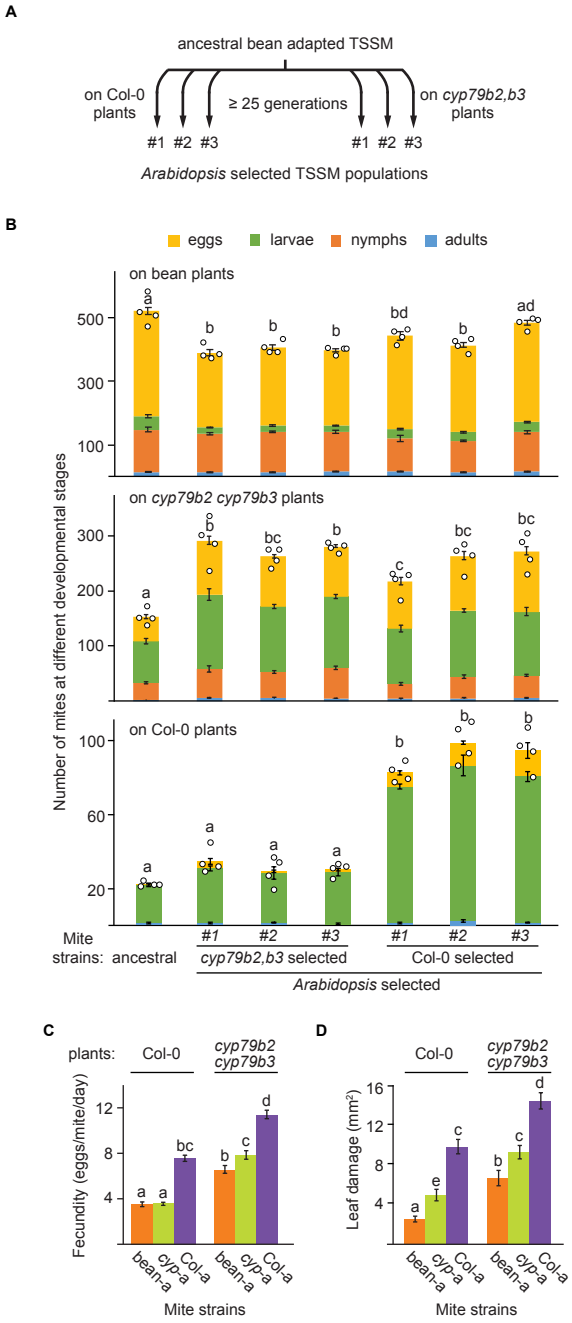

A
ancestral bean adapted TSSM
on Col-0 plants
≥ 25 generations
on cyp79b2,b3 plants
#1 #2 #3
#1 #2 #3
Arabidopsis selected TSSM populations
B
eggs
larvae
nymphs
adults
on bean plants
on cyp79b2 cyp79b3 plants
on Col-0 plants
Number of mites at different developmental stages
Mite strains:
ancestral
#1 #2 #3
cyp79b2,b3 selected
#1 #2 #3
Col-0 selected
Arabidopsis selected
C
plants:
Col-0
cyp79b2 cyp79b3
Fecundity (eggs/mite/day)
bean-a
cyp-a
Col-a
Mite strains
D
Col-0
cyp79b2 cyp79b3
Leaf damage (mm²)
bean-a
cyp-a
Col-a
Mite strains

Figure 2

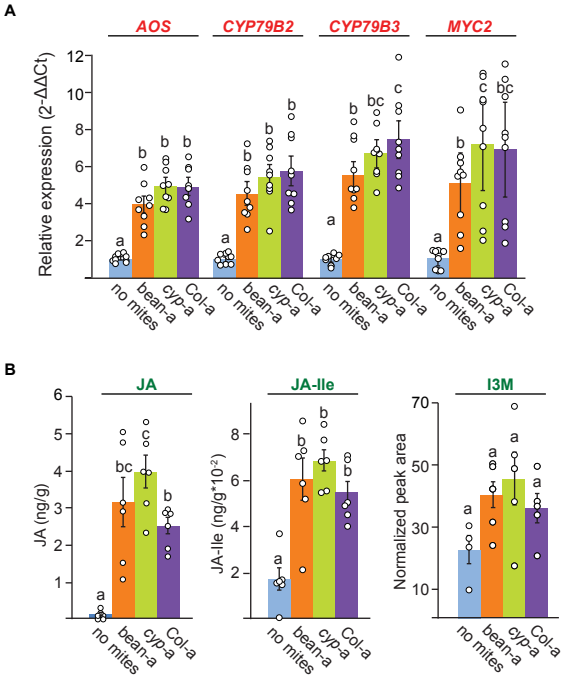

A
AOS
CYP79B2
CYP79B3
MYC2
Relative expression (2-ΔΔCt)
no mites
bean-a
cyp-a
Col-a
B
JA
JA-Ile
I3M
JA (ng/g)
JA-Ile (ng/g*10-2)
Normalized peak area

Figure 3

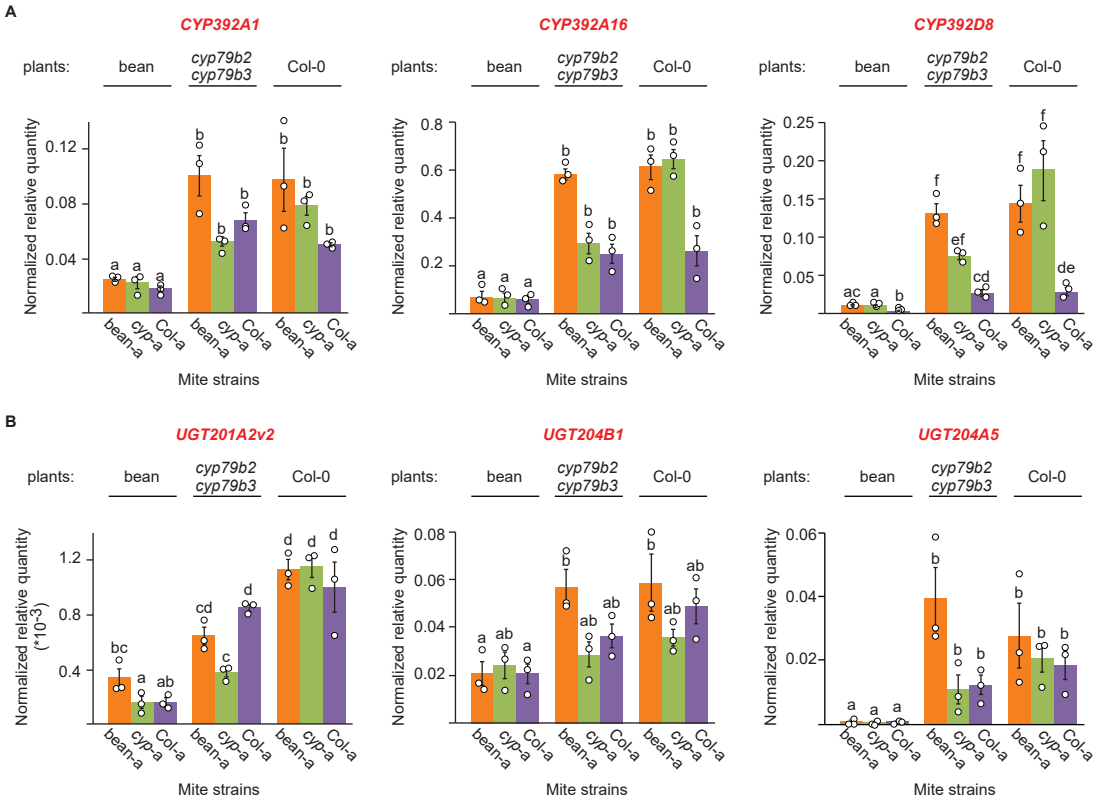

A
CYP392A1
CYP392A16
CYP392D8
plants:
bean
cyp79b2
cyp79b3
Col-0
Normalized relative quantity
Mite strains
bean-a
cyp-a
Col-a
B
UGT201A2v2
UGT204B1
UGT204A5
Normalized relative quantity (*10-3)

Figure 4

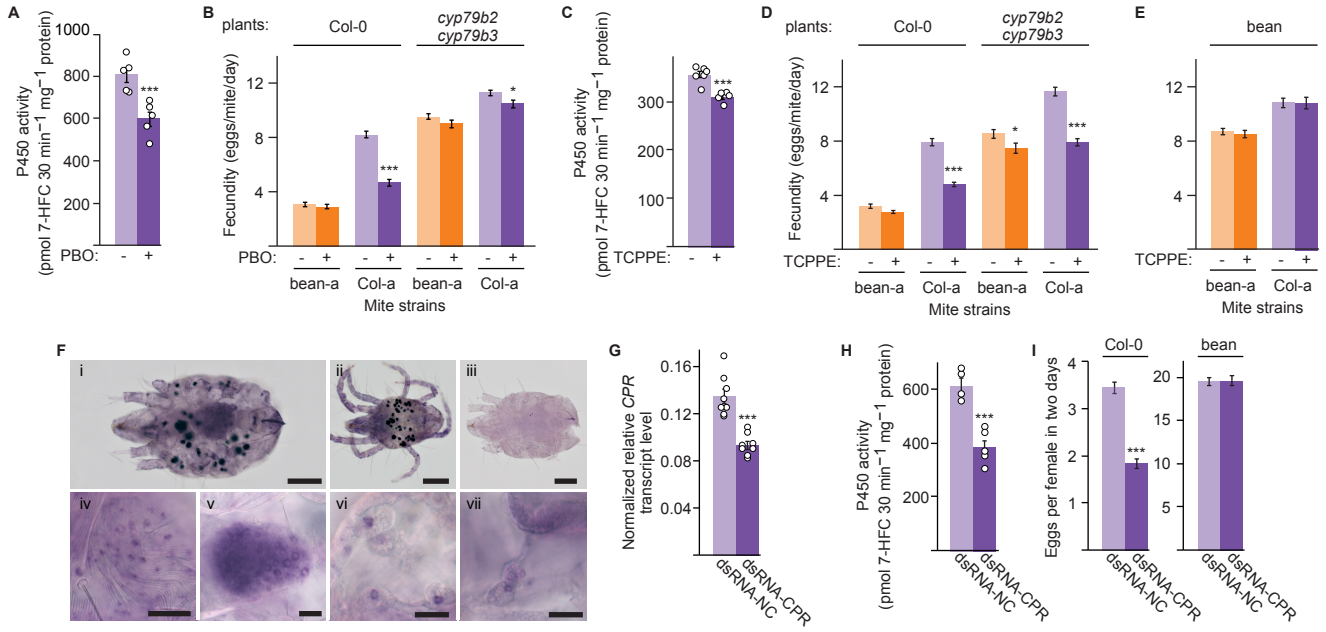
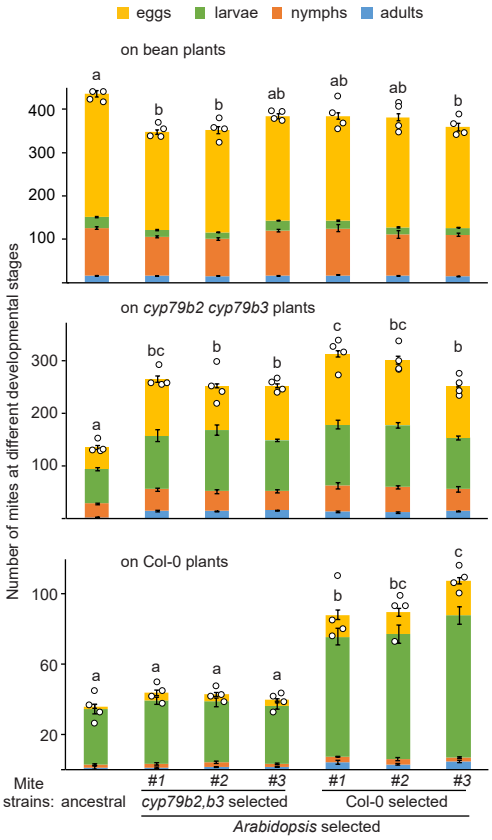
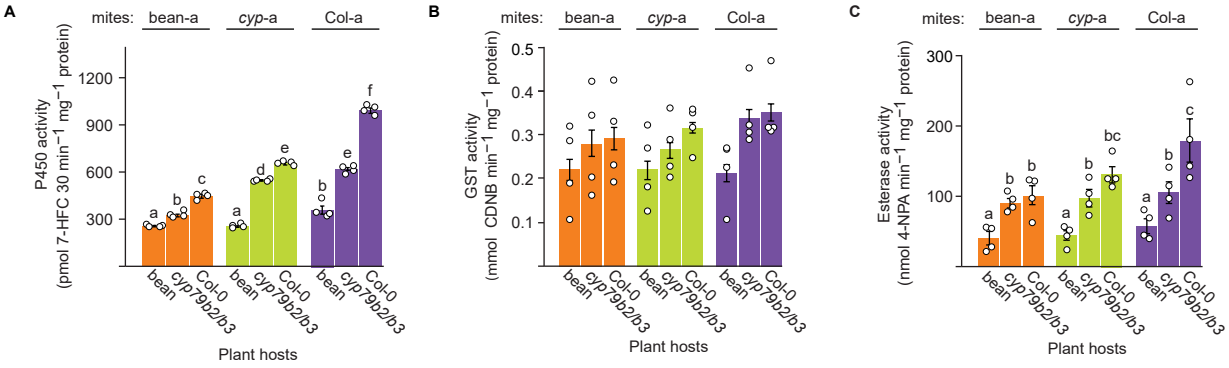

A
P450 activity
(pmol 7-HFC 30 min−1 mg−1 protein)
1000
800
600
400
200
PBO: - +
***
B
plants:
Col-0
cyp79b2
cyp79b3
Fecundity (eggs/mite/day)
12
8
4
PBO: - + - + - + - +
bean-a
Col-a
bean-a
Col-a
Mite strains
***
*
C
P450 activity
(pmol 7-HFC 30 min−1 mg−1 protein)
300
200
100
TCPPE: - +
***
D
plants:
Col-0
cyp79b2
cyp79b3
Fecundity (eggs/mite/day)
12
8
4
TCPPE: - + - + - + - +
bean-a
Col-a
bean-a
Col-a
Mite strains
***
*
***
E
bean
12
8
4
TCPPE: - + - +
bean-a
Col-a
Mite strains
F
i
ii
iii
iv
v
vi
vii
G
Normalized relative CPR
transcript level
0.16
0.12
0.08
0.04
***
dsRNA-NC
dsRNA-CPR
H
P450 activity
(pmol 7-HFC 30 min−1 mg−1 protein)
600
400
200
***
dsRNA-NC
dsRNA-CPR
I
Eggs per female in two days
Col-0
bean
4
3
2
1
20
15
10
5
***
dsRNA-NC
dsRNA-CPR
dsRNA-NC
dsRNA-CPR

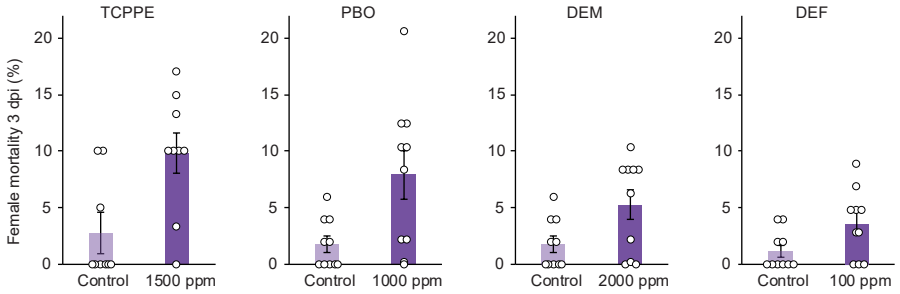
Figure 5



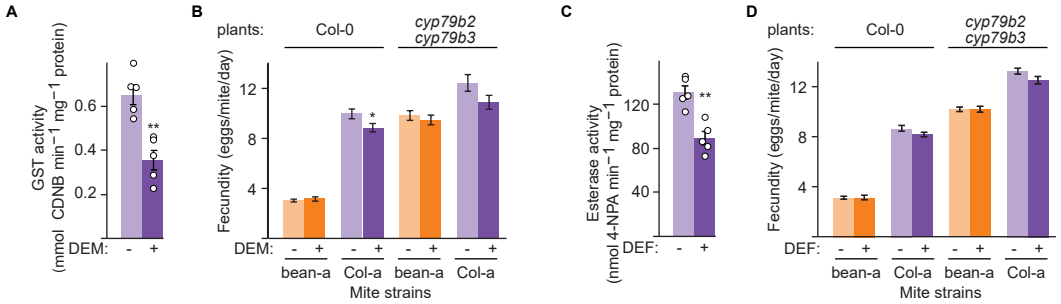
Supplemental Figure S1



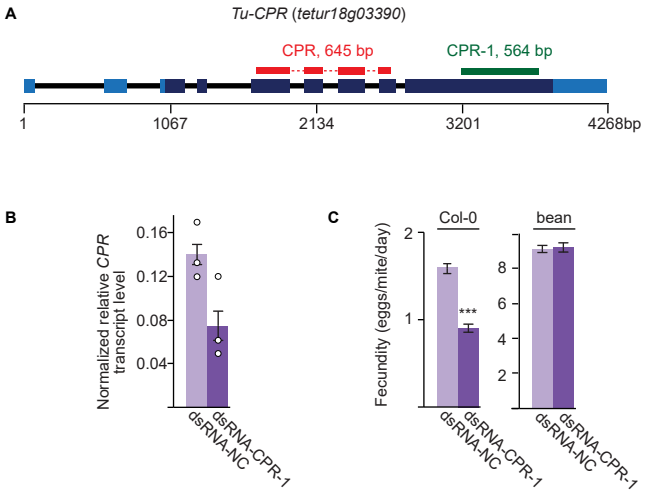
Supplemental Figure S2



Supplemental Figure S3



Supplemental Figure S4



Supplemental Figure S5

Supplemental Table 1. Gene-specific primer sequences used for the analysis of levels of gene expression (RT-qPCR), synthesis of dsRNA (RNAi) and preparation of probe for the *in situ* transcript localization.

Locus ID and name	Application	Primers	Efficiency
tetur07g06410 *CYP392A1*	RT-qPCR	F: 5'-GACGCCTTCGCAAATGATGC-3' R: 5'-TAACCGTCTGTGTTCACGCC-3'	0.929
tetur06g04520 *CYP392A16*	RT-qPCR	F: 5'-TTGATTGGGCTTGCCCTCTT-3' R: 5'-AGCCAACAATCGGAAGACCC-3'	0.903
tetur03g05070 *CYP392D8*	RT-qPCR	F: 5'-ACCAGAGAGATTCCTCAGCG-3' R: 5'-AAAGCCAAAGTTGCACCAGG-3'	1.030
tetur02g02480 *UGT201A2v2*	RT-qPCR	F: 5'-TCGAGAACTGAGTGGAGTAGC-3' R: 5'-TAGCAGGCAAAGGTGTTCCA-3'	1.090
tetur02g09830 *UGT204B1*	RT-qPCR	F: 5'-GCTTCGGTTGAGAAACGTGG-3' R: 5'-AAAATCGGCATTCGCTTCGG-3'	1.000
tetur05g00090 *UGT204A5*	RT-qPCR	F: 5'-TGGACGGAAATCGTAGTGGA-3' R: 5'-AGCTCATCAAAGACCAGCGA-3'	1.090
tetur18g03590 *RP49*	RT-qPCR	F: 5'-CTTCAAGCGGCATCAGAGC-3' R: 5'-CGCATCTGACCCTTGAACTTC-3'	0.976
AT5G42650 *AOS*	RT-qPCR	F: 5'-AAATCCAACGGCGGAGAACT-3' R: 5'-TCGTCGCCAACGGTTGATAA-3'	0.984
AT4G39950 *CYP79B2*	RT-qPCR	F: 5'-GAAAAGAGGTTGTGCGGCTC-3' R: 5'-TCTCACTTCACCGTCGGGTA-3'	0.994
AT2G22330 *CYP79B3*	RT-qPCR	F: 5'-TCTACCGATGCTTACGGGATTG-3' R: 5'-TACAAGTTCCTTAATGGTTGGTTTG-3'	0.973
AT1G32640 *MYC2*	RT-qPCR	F: 5'-TCGCTTACATCAACGAGCTTAAATC-3' F: 5'- TATCTTCACTTCAATCTCCATCCCC-3'	0.900
AT5G25760 *PEROXIN4*	RT-qPCR	F: 5'-GCTCTTATCAAAGGACCTTCGG-3' R: 5'-CGAACTTGAGGAGGTTGCAAAG-3'	0.992
tetur18g03390 *Tu-CPR*	RT-qPCR	F: 5'-CCATTCTTGGCACCTATCGT-3' R: 5'-GCAAGGTGATCTCCAGCTTC-3'	0.994
tetur18g03390 *Tu-CPR*	RNAi (fragment 1)	F: 5'-[T7]-CCTCGACTTCAGCCACGTTA-3' R: 5'-[T7]-AACATCCCGAGCCATGTTCC-3'	not applicable
tetur18g03390 *Tu-CPR*	RNAi (fragment 2)	F: 5'-[T7]-ACAAACCGGTACTGCAGAGG-3' R: 5'-[T7]-TGCATACACGAACGGTCTCC-3'	not applicable
T. urticae genomic scaffold 12, position 1690614-1690995, *NC*	RNAi (negative control)	F: 5'-GCCCTCTCCTGGTTGTAAACTT-3' R: 5'-CGACCCCATCAGGCTATTGA-3'	not applicable
tetur18g03390 *Tu-CPR*	*in situ* (antisense)	F: 5'-[T7]-ACAAACCGGTACTGCAGAGG-3' R: 5'-TGCATACACGAACGGTCTCC-3'	not applicable
tetur18g03390 *Tu-CPR*	*in situ* (sense)	F: 5'-ACAAACCGGTACTGCAGAGG-3' R: 5'-[T7]-TGCATACACGAACGGTCTCC-3'	not applicable

[T7]: T7 RNA Polymerase promoter sequence, 5'-TAATACGACTCACTATAGGG-3'